

DEVELOPMENT OF RABBIT AND CATTLE
OVA IN VITRO

by

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A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfilment of the requirements for the
degree of Master of Science

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July 1969

ABSTRACT

M.Sc.

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Animal Science
(Physiology)

DEVELOPMENT OF RABBIT AND CATTLE OVA IN VITRO

Trials involving 178 follicular rabbit oocytes were conducted to study the importance of the supporting cumulus oophorus and corona radiata cells in oocyte maturation. Further trials involving 539 follicular rabbit and 306 follicular cattle oocytes were conducted to study the influence of the ovarian hormones, progesterone and estradiol 17β on oocyte maturation. Removal of the supporting cells from the oocyte or incubation of the oocyte in the presence of estradiol 17β inhibited resumption of meiosis and oocyte maturation. The inhibition observed by removal of supporting cells was overcome by the re-addition of cumulus cells or incubation of the oocyte in the presence of progesterone. Fertilization of follicular rabbit oocytes culture in vitro was achieved following transfer into a recipient female. Trials involving 283 two-celled rabbit ova were conducted to develop a reliable method by which such ova would continue to cleave in vitro. One hundred per cent serum was observed to be a suitable culture medium.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. R.D. Baker for his direction, guidance, and assistance during the course of the research and preparation of this thesis.

I also wish to thank E.G. Coggins, H.I. Lawson, B. F. Shea, R. Channon, K. Bedirian, and D. Parkinson for their aid in conducting various aspects of the research. Thanks are also extended to Mrs. J. Vauthier who, very conscientiously, typed the final manuscript.

Last, but certainly not least, I wish to express my appreciation to my wife, Elaine, whose patience, understanding, and encouragement served as a source of motivation throughout the past two years.

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INTRODUCTION

Studies concerned with the development of mammalian ova in vitro have been restricted by the difficulty in obtaining sufficient numbers of viable oocytes and by the failure of the oocytes to survive prolonged in vitro manipulation. In the past, research involving gametes has been largely concerned with the preservation and physiology of spermatozoa. More recently however, increased emphasis has been placed on the recovery and incubation of ova.

Superovulation procedures have been used to increase the number of ova ovulated in laboratory and domestic animals. Even though these procedures increase the numbers of ova ovulated, the ova are often lost during recovery. Thus, many oocytes within the ovary which are potentially available are still not utilized.

Techniques by which oocytes are removed from their ovarian follicles and are cultured in vitro have significantly increased the availability of viable ova. Development of efficient in vitro systems for storing, culturing, fertilizing, and growing fertilized ova will enable researchers to study the early stages of embryonic development,

genetically regulated diseases, sex determination, and many other areas involving ova. Efficient methods of transferring unfertilized or fertilized ova into recipient females will make possible routine shipments of ova to distant locations, reducing the costly need to ship and quarantine large animals. Ova from animals of superior quality could be transferred into lesser quality animals for completion of embryonic and fetal development. These and many other uses require reliable in vitro techniques for manipulating and culturing ova. Work to date has not fully elucidated the factors involved in the development of ova. Much emphasis has been placed on various techniques and culture media but relatively few reports have been concerned with such things as the factors which induce oocyte maturation in vivo and in vitro and the influences that the follicle or the oocyte itself have on maturation. Much research remains to be done before efficient use can be made of the large supply of ovarian oocytes.

The objectives of the experiments reported in the thesis were:

- a) To develop a reliable system for culturing follicular oocytes.
- b) To study the importance of the supporting cumulus

ooporus and corona radiata cells on oocyte maturation in vitro.

c) To study the influence of progesterone and estradiol 17 β on oocyte maturation in vitro.

d) To determine whether any relationship exists between the supporting cells and these steroid hormones and oocyte maturation.

e) To determine the fertilizability of in vitro cultured oocytes by transferring into recipient females.

f) To develop a reliable method for culturing two-celled ova in vitro.

REVIEW OF LITERATURE

Maturation of Follicular Oocytes

Stages of maturation of follicular oocytes have been reviewed by Edwards (1966). The first four stages of the first meiotic division occur in the ovary of the developing fetus. Having completed the leptotene, zygotene, pachytene and diplotene stages, meiosis stops. A nucleus, referred to as a germinal vesicle, or vesicular nucleus forms at this point. The oocyte gradually becomes enveloped by cells known as granulosa cells. These cells in turn form the follicle in which the oocyte remains until ovulation. Following the onset of sexual maturity, follicular growth is brought about by the release of follicle stimulating hormone (FSH) from the pituitary gland. This growth is characterized by an increase in the number of granulosa cells and the eventual formation of a fluid filled cavity or antrum. As the size of the antrum increases, the follicle becomes a Graafian follicle. The Graafian follicle is responsive to pituitary luteinizing hormone (LH) which, when released, results in resumption of meiosis of the oocyte and ovulation or

release of the oocyte from the follicle. Upon resumption of meiosis, the first meiotic division is completed, having progressed through diakinesis, metaphase, anaphase, and telophase. One of the daughter cells receives a very small proportion of cytoplasm and is known as the first polar body. The second meiotic division begins as soon as the first meiotic division is completed, and proceeds quickly to metaphase. In most mammals, ovulation of the oocyte occurs while it is in this second metaphase.

Much of what is known about animal reproduction and embryonic development has been learned from such organisms as the chicken and the frog, partly because their ova are readily available for investigation. Similarly, the large numbers of the male gamete have greatly assisted in their being studied. In mammals, the female gamete is not readily available in sufficient numbers. Superovulation, while increasing the number of oocytes released into the oviducts does not significantly improve the availability of the oocytes.

Normal mammalian ovaries contain several thousand oocytes all of which are preformed by the time of birth. Erickson (1966) estimated as high as 700,000 oocytes in some bovine ovaries although the mean was estimated to be

between 50,000 and 100,000 oocytes. Approximately 500,000 oocytes were found in some human ovaries (Edwards, 1966) and up to 1,260,000 germ cells were estimated in the pre-natal pig ovary (Black and Erickson, 1968). Although estimates vary, it is well demonstrated that the ovary could provide large numbers of oocytes for experimental studies.

Pincus and Enzmann (1935) found that rabbit oocytes would mature in vitro when liberated from their follicles into a suitable culture medium. Thus, they demonstrated that luteinizing hormone is not required to effect oocyte maturation in vitro as it is in vivo. To date, successful in vitro maturation of follicular oocytes has been achieved in several species including the mouse (Edwards, 1962; 1965b; Biggers et al., 1967; Donahue, 1968; Donahue and Stern, 1968), the rat (Edwards, 1962), the hamster (Edwards, 1962), the pig (Edwards, 1962, 1965b), the sheep (Edwards, 1962, 1965b), the cow (Edwards, 1962, 1965b; Sreenan, 1968), the rhesus monkey (Edwards, 1962, 1965b; Suzuki and Mastroianni, 1966, 1968a), and the human (Pincus and Saunders, 1939; Edwards, 1965a,b; Edwards et al., 1966) Chang (1955a,b) matured rabbit oocytes in vitro and demonstrated that such oocytes were capable of being fertilized

when transferred into the fallopian tubes of an inseminated recipient rabbit.

Although maturation in vitro of the follicular oocytes was achieved in these various species, the degree of success varied, depending on the specie and the techniques used. A brief summary of some of the results obtained, times of incubation, and media used by several workers is outlined in Table 1. Relative success in the table includes either the incidence of germinal vesicle breakdown or the incidence of complete maturation. The latter describes oocytes in which first polar bodies were observed, whereas, germinal vesicle breakdown (GVBD) describes those oocytes which matured beyond the germinal vesicle or vesicular nucleus stage but not necessarily to the first polar body stage. Ovarian oocytes are in this germinal vesicle stage either at or shortly following birth.

The fact that follicular oocytes resume meiosis in vitro as a result of being removed from their follicles is indeed very interesting in view of the fact that maturation of oocytes in vivo occurs concomittant with LH release and ovulation. The exact mode of action of LH or exogenous hormones with a similar action has not yet been elucidated. Apparently, LH does not influence the oocyte

Table 1. Summary of literature concerned with maturation of mammalian oocytes in vitro

Authors	Species	Incubation Time	Media	Relative Success
Chang, 1955a	Rabbit	11 hours	Ringer-Lockes plus 50% Serum	75-86% GVBD
Edwards, 1965b	Mouse	15-18 hours	TC 199 + Serum	80% GVBD
Biggers <u>et al.</u> , 1967	Mouse	16-19 hours	Hanks Saline + Serum	87% maturation
Donahue, 1968	Mouse	11-17 hours	Krebs-Ringer plus pyruvate	85% GVBD
Edwards, 1965b	Pig	43-46 hours	Krebs-Ringer	80% maturation
Edwards, 1965b	Rhesus Monkey	40 hours	TC 199 + Serum	70% GVBD
Suzuki and Mastroianni, 1966	Rhesus Monkey	46 hours	Waymouth's + Serum	80% GVBD
Edwards, 1965a	Human	40 hours	TC 199 + Serum	80% maturation
Edwards <u>et al.</u> , 1966	Human	40 hours	TC 199 + Serum	70% GVBD
Edwards <u>et al.</u> , 1969	Human	38 hours	TC 199 + Serum	70% maturation
Edwards, 1965b	Cow	31 hours	TC 199 + Serum	100% maturation (14 oocytes)
Sreenan, 1968	Cow	30 hours	Eagles Medium + Serum	90% maturation

directly, since duplication of the maturation process is achieved in vitro in the absence of LH.

Edwards (1965a) reported that follicular oocytes mature synchronously in vitro, even though taken from ovaries in widely different endocrinological stages, thus making it possible to predict the stage of development after a certain period of culture.

Studies with the follicular oocytes of the frog, Rana pipiens, demonstrated that maturation can also be achieved in vitro by removing the oocytes from their follicles (Smith et al., 1968; Subtelny et al., 1968). The ovarian hormone, progesterone was implicated in the stimulation of the maturation processes in frogs (Schuetz, 1967a,b; Masui, 1967). Previously, progesterone had been shown to have an important effect on stimulating ovulation in the frog (Berger and Li, 1960; Wright, 1961; Edgren and Carter, 1963). Berger and Li (1960) were able to cause ovulation by adding excised frog ovaries to Holtfreeters solution containing progesterone at a minimum concentration of 0.6 $\mu\text{g/ml}$. They also demonstrated that estrogens were not capable of causing such in vitro ovulation. Estrogen, unlike progesterone, was not capable of inducing germinal vesicle breakdown in amphibian

oocytes (Schuetz, 1967b). It has been suggested that the hormonal induction of germinal vesicle breakdown with pituitary hormones and progesterone is mediated by an intra-follicular synthesis of protein. Puromycin, a specific inhibitor of protein synthesis, blocked the action of luteinizing hormone and progesterone by completely inhibiting maturation, suggesting that protein synthesis is involved in the hormonal induction of germinal vesicle breakdown (Schuetz, 1967a; Brachet, 1967).

Ovulation studies in mammals and birds revealed that ovulation is induced under the influence of both pituitary luteinizing hormone and progesterone. Administration of luteinizing hormone to immature rabbits caused ovulation and stimulated the ovarian production of progesterone (Hilliard et al., 1961). Although progesterone induced ovulation, the effect was dependent on pituitary activity, since removal of the pituitary abolished the effect of progesterone (Sawyer and Everett, 1959). The action of progesterone in facilitating ovulation was demonstrated in several animals including the hen (Fraps, 1943), rhesus monkey (Pfieffer, 1950), rat (Everett, 1944) and rabbit (Sawyer et al., 1950).

The precise mechanism of action of progesterone

concerning the ovulatory processes has not been reported. The influence of the follicular cells surrounding the oocyte of Rana pipiens was reported by Masui (1967). He demonstrated that oocytes would not undergo maturation in vitro in the presence of LH when the follicular cells were removed. Luteinizing hormone induced oocyte maturation when the follicular cells were re-added, indicating that the pituitary hormone may act on the follicle cells, causing them to produce a factor which, in turn, acts on the oocyte to induce maturation. Schuetz (1967b) demonstrated that the germinal vesicle breakdown of amphibian oocytes which occurred in vitro in the absence of LH was inhibited by removing the supporting cells surrounding the oocyte. Progesterone was able to cause germinal vesicle breakdown even after removal of the supporting follicle cells, indicating that progesterone acts directly on the frog oocyte to induce maturation (Masui, 1967).

No reports were found concerning the effect of the steroids estrogen and progesterone on the maturation of mammalian oocytes. Estrone and estradiol 17β have been isolated from ovarian tissue (Dorfman and Ungar, 1963) and from follicular fluid (Smith, 1960). Androgens have been found in ovarian tissue (Zandler, 1958) and have been

shown to be precursors to estrogen (Wotiz et al., 1956). However, there is no evidence that androgens are normally secreted, although certain pathological conditions result in androgen secretion (Scully, 1963). Edgar (1953) reported the presence of progesterone in the follicles of cows and sows prior to ovulation. The presence of progesterone in follicular fluid was also reported by Zandler (1954).

Hilliard et al. (1961, 1963) and Solod et al. (1966) demonstrated increases in the synthesis and secretion of progesterone from rabbit ovaries shortly following LH or human chorionic gonadotrophin stimulation, yet prior to ovulation. Similar increases in progesterone levels were observed in rats (Eto et al., 1962). A significant decrease in the synthesis of estradiol 17 β by the ovaries of proestrus and estrus rats was observed following LH stimulation (Chatterton et al., 1969). They suggested that the effect of LH on the mature follicle could be to decrease estrogen synthesis by the process of luteinization.

Channing (1966) incubated equine granulosa cells from follicles of ovaries collected at various stages of the estrus cycle and demonstrated that, regardless of the

stage of the estrus cycle, the granulosa cells took on morphological features characteristic of luteal cells, irrespective of whether gonadotrophin was added to the medium. She demonstrated that these cells were capable of synthesizing steroids. During a 7-day period, 340 μ g of progesterone, 35 μ g of 17 α hydroxyprogesterone, 89 μ g of hydroxy pregn 4 en 3 one 20 α , and 35 μ g of androstenedione were synthesized by 20,000 to 40,000 live cells as compared to only a trace of estrogen.

Follicular oocytes found denuded of the surrounding granulosa cells within the follicle are generally atretic and often meiosis in such oocytes has advanced beyond the germinal vesicle stage. Thus, the possibility arises that LH of the previous cycle may have stimulated luteinization of the granulosa cells which surround the oocyte resulting in atresia. Erickson (1966) reported that the incidence of follicular atresia in the bovine increases after the age of six months.

Follicular atresia may have its onset at any stage of follicular growth or maturation, and oocytes may degenerate before they have acquired a distinct membrana granulosa (Mandl and Zuckerman, 1950). The actual cause of follicular atresia is not known. In rats, the increased

incidence of atresia which occurs post ovulation has been attributed to the action of the corpora lutea (Atkinson and Leathem, 1946). Administration of progesterone to rats increases the incidence of atresia, whereas the administration of estrogen demonstrates the opposite effect (Young, 1961). Characteristics of an atretic follicle, dissolution of the granulosa cells and denudation of the oocyte, have led to the suggestion that the stimulus for normal ovulation is also involved in follicular atresia, since, besides becoming denuded of surrounding follicular cells, the oocyte within the atretic follicle often matures to the stage seen at ovulation (Edwards, 1965a; Suzuki and Mastroianni, 1966). In conclusion it appears that changes in the ova during atresia and ovulation result from hormonal changes associated with these phenomena. There appears to be a relationship between the increased progesterone production, and changes in the granulosa cells following LH stimulation: dissolution of the granulosa cells occurs and the oocyte matures to complete the first meiotic division, and metaphase of the second meiotic division. Studies with frog oocytes demonstrated that the granulosa cells surrounding the follicular oocyte are necessary for maturation in vivo and in vitro. Removal of

these cells inhibits maturation. Progesterone, however, is capable of inducing maturation of these denuded oocytes.

In mammals, increased levels of progesterone and decreased levels of estrogen have been demonstrated in preovulatory follicles following LH stimulation. Incubation of equine granulosa cells has shown that they are capable of synthesizing large amounts of progesterone. Progesterone and granulosa cells have been implicated in follicular atresia and the partial or complete maturation of the oocyte which occurs in such follicles.

Fertilization of oocytes cultured *in vitro*

Once the oocyte has extruded the first polar body and has advanced to metaphase of the second meiotic division, it is considered to be ready for fertilization. Only when the fertilizing spermatozoon has entered the oocyte is the second meiotic division ended by extrusion of the second polar body (Edwards, 1966).

During the past few years, extensive research has been carried out to develop suitable techniques for the fertilization of oocytes *in vitro*. It appears that in most mammalian species studied, the spermatozoa must be exposed to the female reproductive tract for a period of

time before they are capable of fertilizing oocytes. During this time, the spermatozoon undergoes certain changes, known as capacitation. These changes primarily involve the acrosome cap of the sperm cell. Freshly ejaculated rabbit spermatozoa have been found to be incapable of penetrating through the zona pellucida of the oocyte. A six-hour period of incubation in the female tract was found to be necessary before the rabbit spermatozoa could penetrate the oocyte (Austin, 1951; Chang, 1951). This has been an important factor in the fertilization of rabbit oocytes in vitro. Success of such experiments has depended on the use of spermatozoa recovered from the female reproductive tract (Thibault and Dauzier, 1961; Dauzier and Thibault, 1961; Chang, 1959; Bedford and Chang, 1962; Brackett and Williams, 1965, 1968; Suzuki and Mastroianni, 1965, 1968b). In hamsters, the process of capacitation appears to be unnecessary (Yanagimachi and Chang, 1964). The need for capacitation has been demonstrated in the rat, although the time required is much shorter. When rat spermatozoa were introduced at laparotomy into the oviduct, fertilization was not initiated until four hours later (Austin, 1951). Attempts to capacitate spermatozoa in vitro have generally been unsuccessful. However,

capacitation of rabbit spermatozoa in vitro has been reported with the use of the enzyme β amylase (Kirton and Hafs, 1965), and with the use of mule eosinophils (Ericsson, 1969). Much work remains to be done, however, with the rabbit and with other mammalian species in order to elucidate the processes involved in capacitation and to develop reliable methods of overcoming the phenomenon in order to facilitate and improve in vitro fertilization techniques.

To date, the most widely used method of fertilizing oocytes which have been cultured in vitro is to transfer them into the reproductive tract of a recipient female which has been inseminated with spermatozoa. The oocytes are transferred into the oviduct, the site of normal fertilization. Attempts to fertilize hamster oocytes following transfer into the uterus were unsuccessful (Hunter, 1968), indicating that transfer into the oviduct is necessary for optimum fertilization. The successful transfer of the unfertilized oocyte has been dependent on surgical techniques, since oocytes must be deposited in the oviduct.

Bovine ova transfers have received much attention, yet relatively few successes have been reported (Willet et al., 1953; Avery et al., 1962; Rowson et al., 1969).

Problems associated with bovine ova transfers have been discussed in detail by Dziuk et al. (1958). Transfer attempts in the past have necessitated the use of ovulated ova. Donors must be superovulated and the ova recovered either by sacrificing the donor or by surgically flushing the ova from the reproductive tract (Rowson et al., 1969). Both of these methods limit the practical usefulness of transferring ova between cattle. It is important to synchronize the estrus cycle of the recipient female to coincide with the development of the ova being transferred (Dziuk et al., 1958; Rowson et al., 1969). The manipulation of ova and the problems associated with such techniques are also discussed by Dziuk et al. (1958).

Pig ova have also been successfully transferred by surgical methods (Hancock and Hovell, 1962; Dziuk et al., 1964; Vincent et al., 1964; Polge, 1966; Hunter et al., 1967). Successful ova transfers have been reported in the sheep (Hunter et al., 1955, 1962; Hancock and Hovell, 1961; Adams et al., 1961; Rowson and Moor, 1966a; Moor, 1968).

A suitable non-surgical method of transferring ova would be advantageous. Such non-surgical techniques are receiving increased emphasis. Successful non-surgical

ova transfers have been reported in the pig (Polge and Day, 1968). Based on a technique first described by Hafez and Sugie (1961), successful non-surgical ova transfers have also been performed on the cow (Mutter et al., 1964; Sugie, 1965; Rowson and Moor, 1966b). The non-surgical technique involves transferring the ovum into the uterus and therefore necessitates that the ovum be fertilized and developed to the stage that would continue to cleave and implant in the uterus.

Fertilized sheep ova (Averill et al., 1955) and fertilized cow ova (Sreenan and Scanlon, 1968) have been transferred into the reproductive tract of the rabbit where they continue to cleave normally up to the blastocyst stage, indicating that the rabbit could potentially serve as an in vivo incubator for the long distance shipment of ova. Such an in vivo system of shipping ova or a reliable in vitro system would be of extreme value. Baker and Dziuk (1969) successfully carried out the first long distance shipment of fertilized pig ova between two countries. For this experiment they used an in vitro system employing a synthetic tissue culture medium (Medium 199). A time interval of ten hours was involved in the shipment of the ova.

In summary, fertilization of oocytes of most mammalian species studied requires that the spermatozoa be exposed to the environment of the female reproductive tract for a period of time. Capacitation of the spermatozoa which occurs during this period is not completely understood. It serves as a major limiting factor to the fertilization of oocytes in vitro. Fertilization in vivo of follicular oocytes cultured in vitro requires transfer of the oocyte into the oviduct of the recipient. Because of the surgical procedures involved a greater future lies in the non-surgical transfer of fertilized ova. Such procedures necessitate the use of ova in the early cleavage stages.

Cleavage of Fertilized Ova "In Vitro"

The fact that current non-surgical ova transfer methods require the transfer of fertilized, pre-implantation embryos, suitable techniques of cleaving ova in vitro are necessary if maximum use is to be made of ova which have been cultured and fertilized in vitro. Such techniques would be advantageous in the study of early embryonic development and would facilitate sex determination of these early embryos (Gardner and Edwards, 1968).

Several investigators have reported successful attempts at culturing fertilized ova in vitro from the rabbit (Smith, 1949; Chang, 1950; Purshottam and Pincus, 1961; Daniel, 1964a,b, 1965; Edwards, 1964; Daniel and Cowan, 1966; Daniel and Krishnan, 1967; Staples, 1967; Daniel and Olson, 1968; and Onuma et al., 1968), the cow (Thibault, 1966; Sreenan et al., 1968), and the mouse (Whitten, 1956; McLaren and Biggers, 1958; Biggers et al., 1962; Brinster, 1963, 1965a,b,c,d, 1968; Brinster and Thomson, 1966; Gwatkin, 1966; Auerbach and Brinster, 1968; Whitten and Biggers, 1968; TenBroeck, 1968; Wales and Brinster, 1968). Most of the studies have been concerned with the development of suitable media for cleaving ova. First cleavage of rabbit ova occurred in an amino acid free medium but the second cleavage required the presence of the amino acids cysteine, tryptophane, phenylalanine, lysine, arginine, and valine. Subsequent cleavage required the addition of methionine, threonine, and glutamine to the medium (Daniel and Olson, 1968). The five-day-old rabbit blastocyst has an essential requirement for ten amino acids, including three which were not required for earlier cleavages (Daniel and Krishnan, 1967). Detailed studies on the cleavage of mouse ova have indicated an

optimum pH of 6.8 (Brinster, 1965a). Energy requirements, amino acid requirements, and the effects of fixed nitrogen sources have been studied (Brinster, 1965a,b,c,d; Gwatkin, 1966). Such studies have demonstrated that the requirements for cleavage vary not only according to species but also according to the stage of cleavage of the ovum.

Prolonged exposure to visible light or short exposure to ultraviolet light has an inhibitory effect on the cleavage of mammalian ova (Daniel, 1964b).

Attempts to cleave mammalian ova in vitro from the early stages through to the blastocyst stage have largely been unsuccessful, probably due to the different requirements of the developing stages. Rabbit and bovine serum are capable of supplying the necessary requirements for the development of two- and four-celled rabbit ova into blastocysts (Onuma et al., 1968). Treating the ova with one per cent pronase solution to weaken the zona pellucida and adding 0.05 per cent glucose to the serum to serve as an extra source of energy significantly increased the number of ova developing to the blastocyst stage. Ova were cultured in Falcon tissue culture dishes in droplets of medium under paraffin oil at 36°C in a humidified mixture of 5% CO₂:95% air. A similar technique was used to

culture two-celled mouse ova through to the blastocyst stage in vitro (Brinster, 1963).

There is a great potential for the shipment of fertilized ova to distant locations. To date, there is no adequate in vitro system by which fertilized ova can be maintained for the periods of time required for such shipments. However, fertilized cow and sheep ova have been shown to continue cleaving in the reproductive tract of a female rabbit (Hunter et al., 1962; Adams et al., 1968; Sreenan and Scanlon, 1968). Such a system could serve as a useful method of shipping fertilized ova which have been cultured in vitro until a reliable method has been developed by which a completely in vitro system may be used in the shipment of ova. Such ova would then be transferred into recipient females for completion of the embryonic and fetal development.

MATERIALS AND METHODS

Rabbits, cattle, and swine ovaries were obtained either by ovariectomy or immediately after slaughter. Virgin New Zealand White does, weighing 2.8 to 4.4 Kg, were used as a source of rabbit ovaries. The majority of cow ovaries were recovered from mature animals killed at a commercial abattoir. Swine ovaries were removed from gilts weighing 85 to 95 Kg. These gilts were slaughtered at Macdonald College.

Oocytes were removed from the ovarian follicles using the dissecting equipment shown in Figure 1. The follicle was cut or punctured and a slight pressure applied in order to force out the follicular fluid and the oocyte. The rabbit and the swine oocytes were placed immediately in the incubation medium. Glass vials were used to transport the cattle follicular fluid and oocytes from the abattoir to the laboratory, a distance of approximately 25 miles. Temperature was maintained by placing the glass vials in a one-gallon thermos containing water at 37°C. At the laboratory the oocytes were then transferred from the follicular fluid to the incubation medium. A stereoscopic microscope was used to assist in the manipulation

of the oocytes (Figure 3).

All oocytes were incubated in sterile Falcon plastic dishes (50 mm x 12 mm), in an incubator at 37°C, with 5% CO₂ in high humidity air. The oocyte manipulation equipment and incubation dishes are shown in Figure 2. A few oocytes were retained, at each incubation, to serve as controls. These oocytes were immediately observed and fixed to determine the stage of maturation.

Following incubation, the oocytes were transferred from the culture dishes to small tubes and shaken vigorously in physiological saline for about one minute in order to remove the corona radiata and cumulus oophorus cells which surround the oocyte (Figure 4), thus facilitating observation of the oocyte. The oocytes, in a drop of physiological saline, were placed on a microscope slide between two ridges of a mixture of paraffin-stopcock grease. A coverslip was gently pressed down on the ridges until contact was made with the saline drop. The oocytes were then examined for general condition and presence or absence of the first polar body. The oocytes were then fixed for 48 hours in 30 per cent acetic acid-70 per cent alcohol, and stained with 0.5 per cent orcein in 40 per cent acetic acid. The stained chromatin, examined by

phase contrast and interference contrast microscopy, indicated the stage of maturation of the oocyte.

EXPERIMENTS AND RESULTS

EXPERIMENT I: MATURATION OF FOLLICULAR RABBIT, CATTLE AND SWINE OOCYTES IN VITRO

Experiment I, which consisted of four trials, was carried out with the purpose of studying various factors involved in follicular oocyte maturation in vitro.

Trial 1 was carried out to observe oocytes at the various stages of maturation and to obtain photographs of oocytes at the different stages which would assist in identifying stage of maturation of oocytes of future trials and experiments. Condition of the chromatin was used to indicate the stage of maturation.

Trial 2 was conducted to test the effects of pre-exposing the medium to the conditions provided by the incubator. Two methods of incubating oocytes were also compared. Oocytes were incubated either in Falcon culture dishes containing the medium only or in similar dishes containing droplets of medium under mineral oil.

Trial 3 was conducted to test the in vitro culture system on bovine follicular oocytes and to compare two methods of recovering bovine ovaries, these being surgical

recovery and recovery from animals slaughtered at a nearby abattoir.

Trial 4 was conducted to test the in vitro system of follicular oocyte culture on the maturation of follicular rabbit, cow, and pig oocytes.

TRIAL 1: Nuclear changes during oocyte maturation

Sixty follicular rabbit oocytes were incubated in a medium consisting of 90 per cent TC 199 (Difco, Detroit, U.S.A.), 10 per cent rabbit serum, supplemented with 100 I.U. of penicillin and 100 μ g of streptomycin per ml of medium, and buffered with bicarbonate to a pH of 7.0 to 7.2. Each oocyte was incubated in a droplet of medium under mineral oil. Oocytes were removed at regular intervals and observed under the microscope. Nuclear changes were observed following fixation and staining.

The series of photographs, shown in Figures 6 to 19, show the nuclear changes seen during the trial. The relationship between the stage of maturation and time of incubation is outlined in Table 2. With respect to stage of maturation, each oocyte was classified either as immature, partially mature, or mature. Immature oocytes still possessed a germinal vesicle or vesicular nucleus following incubation;

Table 2. Nuclear changes during rabbit oocyte maturation in vitro

Culture Time (Hours)	Stage of Nucleus	Stage of Maturation
0	Germinal Vesicle (Fig. 6)	Immature
1	Germinal Vesicle (Fig. 7)	Immature
2	Germinal Vesicle (Fig. 8)	Immature
3	Germinal Vesicle (Fig. 9)	Immature
4	Germinal Vesicle (Fig. 10)	Immature
5	Germinal Vesicle (late) (Fig. 11)	Immature
6	Diakinesis - Early Metaphase (Fig. 12)	Partially Mature
7	Metaphase I (Fig. 13)	Partially Mature
8	Anaphase (Fig. 14)	Partially Mature
9	Anaphase - Telophase (Fig. 15)	Partially Mature
10	Telophase (Fig. 16)	Partially Mature
11	Telophase - First Polar Body (Fig. 17)	Mature
12	First Polar Body, Metaphase II (Fig. 18)	Mature
13	First Polar Body, Metaphase II (Fig. 19)	Mature

partially mature oocytes had developed beyond the vesicular nucleus stage but had not formed the first polar body as had the mature oocytes. This classification was used to describe the stage of maturation of oocytes of succeeding experiments and trials.

The vesicular nucleus was observed for up to $5\frac{1}{2}$ hours of culture although changes were occurring within the nucleus (Figures 6 to 11). Condensing of the chromatin (Figure 12) and the formation of the first metaphase plate (Figure 13) was observed to occur after 5 to 8 hours of culture. Anaphase and telophase (Figures 14 to 16) were observed after 7 to 10 hours of culture. Completion of the first meiotic division and extrusion of the first polar body and metaphase of the second meiotic division (Figures 17 and 19) were observed to occur after $9\frac{1}{2}$ to 12 hours of culture. After 12 hours of culture, 4 oocytes were found to possess the first metaphase plate and one oocyte still possessed a vesicular nucleus.

TRIAL 2: Pre-exposure of the medium to the incubator and the effect of incubating oocytes in a droplet of medium under mineral oil

One hundred and sixty-eight follicular rabbit oocytes were incubated for 12 hours in a medium consisting of 90 per

cent TC 199, 10 per cent rabbit serum, supplemented with 100 I.U. penicillin and 100 μ g streptomycin, and buffered with bicarbonate to a pH of 7.0-7.2. The oocytes were incubated in a humidified mixture of 5 per cent CO₂:95 per cent air at 37°C. Seventy-five of the oocytes were incubated in medium which had not been pre-exposed to the environment of the incubator. The remaining 93 oocytes were incubated in a medium which had been allowed to equilibrate with the 5 per cent CO₂ in air. Of the 75 and 93 oocytes, 43 and 45, respectively, were incubated in droplets of medium under mineral oil. Each droplet was approximately 75 μ l in size and contained one oocyte. Falcon culture dishes were used for all incubations with 4 to 6 oocytes being incubated in each dish. The mineral oil used for oocytes being incubated in the pre-exposed medium was also pre-exposed to the incubator.

With the pre-exposed medium, 39 (87%) oocytes incubated under oil were observed to have matured to the first polar body stage (Table 3). This was not significantly different ($P > .05$) from the 45 (94%) oocytes which matured in the dishes containing the medium only. Of the oocytes incubated in the non-pre-exposed medium, there was a similar non-significance in the number of oocytes matured in

Table 3. Pre-exposure of medium and influence of mineral oil on culture of follicular rabbit oocytes in vitro

	Medium Pre-exposed				Medium Not Pre-exposed			
	Partially			Total	Partially			Total
	Immature	Mature	Mature		Immature	Mature	Mature	
Oocytes incubated in droplet of medium under mineral oil	2	4	39*	45	10	10	23**	43
Oocytes incubated in medium only (no mineral oil)	2	1	45*	48	9	7	16**	32
Total	4	5	84*	93	19	17	39**	75

*Significantly different from ** ($P < .05$)

the droplet under oil and the number of oocytes which matured in the dishes containing the medium only.

Pre-exposure of the medium increased ($P < .05$) the number of oocytes which matured to the first polar body stage. This increase was observed with oocytes incubated in medium only and in droplets of medium under mineral oil. Of a total of 93 oocytes incubated in pre-exposed medium, 89 (96%) exhibited germinal vesicle breakdown. Of these 89 oocytes, 94 per cent matured to the first polar body. Of the 75 oocytes incubated in non-pre-exposed medium, 56 (75%) exhibited germinal vesicle breakdown. Of these 56 oocytes, 70 per cent matured to the first polar body stage. A glass electrode pH meter was used to obtain pH values of the media. The pH of the medium without any exposure to the 5 per cent CO_2 of the incubator was observed to be 8.5 whereas the pH of the medium following exposure to the incubator dropped to 7.0 to 7.2. The TC 199 used contained phenol red which turned the colour of the medium from red to yellow if the pH dropped below 7.0. Visual observation served as a reliable method of appraising the medium to determine if equilibration with the incubator had occurred. It was observed that at least 2 hours of incubation was necessary in order for the medium to become equilibrated.

TRIAL 3: Use of oocytes from surgically
recovered cow ovaries vs cow
ovaries obtained at the
slaughter house

Eight ovaries were recovered by ovariectomy from four cycling cows. Each animal was anesthetized with a paravertebral nerve block and laparotomized from the right side. A chain ecrasseur was used to remove the ovaries. With the aid of a stereoscopic microscope, oocytes were recovered immediately after removal of the ovaries.

The technique for recovery of oocytes from slaughter house ovaries has already been discussed. Generally, between 35 and 45 minutes time lapsed between killing of the cattle and evisceration and recovery of the ovaries.

From the eight surgically recovered ovaries, 29 follicular oocytes were incubated, and from the ovaries obtained at the abattoir, 68 follicular oocytes were incubated. All oocytes were incubated for 40 hours in a medium of 85 per cent TC 199 plus 15 per cent bovine serum and supplemented with 100 I.U. of penicillin and 100 μ g of streptomycin per ml of medium. The medium was buffered with bicarbonate to a pH of 7.1.

Of the 29 oocytes recovered following ovariectomy, 4 remained in the vesicular nucleus stage, and the remaining 25 (86%) all matured to the first polar body stage. Of the

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68 oocytes recovered at the abattoir all matured beyond the vesicular nucleus stage, but only 44 (65%) matured to the first polar body stage. The remaining 24 (35%) oocytes matured to metaphase or anaphase but did not extrude the first polar body. Application of a Chi Squared analysis showed that there was no significant difference between the maturation of oocytes from surgically recovered ovaries and the maturation of oocytes from ovaries obtained from the abattoir.

TRIAL 4: Species differences: Rabbits, Cattle and Swine

In order to test the value of the in vitro oocyte culture procedures discussed in the previous trials, oocytes from three different species were incubated. Two hundred rabbit, 70 cattle, and 190 swine follicular oocytes were incubated for 12, 40, and 48 hours, respectively. The rabbit oocytes were incubated in a medium consisting of 90% TC 199 and 10% rabbit serum, whereas the bovine oocytes were incubated in a medium consisting of 85% TC 199 and 15% bovine serum, and the swine oocytes were incubated in TC 199 with up to 20% porcine serum. All media contained 100 I.U. of penicillin and 100 μ g of streptomycin per ml. Bicarbonate was used to buffer the media to a pH of 7.1. All oocytes were incubated in droplets of medium under mineral

oil in a humidified mixture of 5% CO₂:95% air at 37°C.

The results of this trial are summarized in Table 4. Eighty-nine per cent of the rabbit oocytes and 77% of the cattle oocytes matured to the first polar body stage as compared with only 6% polar body formation with the swine oocytes. Seventy per cent of the swine oocytes failed to mature beyond the germinal vesicle stage as compared with 8% and 15% of the rabbit and cattle oocytes, respectively. The number of swine oocytes exhibiting germinal vesicle breakdown and polar body extrusion was significantly lower ($P < .01$) than the number of rabbit and cattle oocytes. There was no significant difference between the maturation behaviour of the rabbit and cattle oocytes.

The various levels of porcine serum used with the TC 199 did not alter the percentage of swine oocytes exhibiting germinal vesicle breakdown and polar body extrusion.

EXPERIMENT II: IMPORTANCE OF THE CUMULUS
OOPHORUS AND CORONA RADIATA
CELLS ON THE MATURATION IN
VITRO OF FOLLICULAR RABBIT
OOCYTES

a. Effect of removal and readdition of
the cumulus and corona cells

Trials involving 178 follicular rabbit oocytes were

Table 4. Maturation in vitro of follicular rabbit, cow, and pig oocytes

	Rabbit	Cow	Pig
No. of oocytes	400	130	190
Culture time (hours)	12	40	48
Incidence of germinal vesicle breakdown and polar body formation (%)			
Completely matured	87*	78*	6**
Partially matured	4	8	24
Immature	9	14	70

*Significantly different from ** ($P < .01$)

conducted to study the importance of the supporting cells on in vitro maturation. Oocytes were removed from their ovarian follicles and were placed in a medium of TC 199 (90%) and rabbit serum (10%), supplemented with 100 I.U. of penicillin and 100 µg of streptomycin per ml and buffered with bicarbonate to a pH of 7.0 to 7.2. All oocytes were incubated in droplets of medium under mineral oil at 37°C in a humidified mixture of 5% CO₂:95% air. The cumulus oophorus and corona radiata cells were left intact on 216 oocytes. Of these oocytes, 154 were incubated immediately after removal from their follicles and 62 were left in the medium but not placed in the incubator for 7-8 minutes while other oocytes were being agitated. Mechanical agitation was used to remove the cumulus oophorus cells from 50 oocytes, leaving only the corona radiata cells intact. The remaining 61 oocytes were exposed to sufficient physical agitation to remove all cumulus and corona radiata cells (complete oocyte denudation). Just prior to incubation cumulus cells were readdded to the droplets of medium containing 23 of the 61 denuded oocytes. All oocytes were incubated for 12 hours.

Delaying incubation for 7-8 minutes resulted in 63% of the 69 oocytes maturing to extrude the first polar body,

whereas, 88% of the 154 oocytes incubated immediately after recovery showed complete maturation (Table 5). This difference was found, by Chi Squared analysis, to be not significant ($P > .05$). A difference was anticipated because a small volume of medium (4 ml) was used to agitate the oocytes. Thus, the temperature of the medium quickly dropped to that of the laboratory and the pH increased to 8.0-8.5 due to the loss of CO_2 to the air.

Removal of the cumulus oophorus, leaving the corona radiata intact, did not significantly alter ($P > .05$) the number of oocytes exhibiting germinal vesicle breakdown (GVBD) and formation of the first polar body. However, removal of both cumulus oophorus and corona radiata cells reduced ($P < .05$) the number of oocytes exhibiting GVBD and polar body formation. Only 7 (18%) of the 38 denuded oocytes completely matured to the first polar body stage. This was lower than the number of non-denuded oocytes that underwent maturation. Re-addition of cumulus oophorus cells to the droplets of medium containing denuded oocytes increased ($P < .05$) the numbers of oocytes exhibiting GVBD and first polar body extrusion. Sixteen (70%) denuded oocytes exhibited GVBD of which 11 oocytes matured to extrude the first polar body when incubated in the presence of re-added cumulus

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Table 5. Influence of oocyte denudation on maturation in vitro of follicular rabbit oocytes

	Immature	Partially Mature	Mature
Control Oocytes			
Incubated immediately	8 (5%)	11 (7%)	135 (88%)
Incubation Delayed	9 (14%)	14 (23%)	39 (63%)
Oocytes with Intact Corona Radiata	5 (9%)	15 (25%)	35 (66%)
Oocytes completely Denuded of surround- ing Cells	25 (66%)	6 (16%)	7 (18%)
Denuded Oocytes with Cumulus Re-added	7 (30%)	5 (22%)	11 (48%)

cells compared with only 13 (34%) denuded oocytes which exhibited GVBD, 7 maturing to the first polar body, when incubated in the droplet without re-added cumulus cells.

b. Effect of incubating intact follicles

In order to observe the influence that the complete follicle exerts on the maturation of the follicular oocyte, 35 follicles were removed from rabbit ovaries and were incubated in a similar medium and under the same conditions as described in part "a" of this experiment.

Following culture for 12 hours, the 35 follicles were punctured to remove the oocytes. Twenty-three oocytes were examined microscopically and all were found to still contain the germinal vesicle. The remaining 12 oocytes were immediately incubated in the culture medium for another 12 hours. Microscopic examination of these oocytes revealed that, after the second culture period, 4 oocytes had exhibited germinal vesicle breakdown and had matured to metaphase of the first meiotic division. The remaining 8 oocytes did not exhibit germinal vesicle breakdown.

EXPERIMENT III: EFFECTS OF ESTRADIOL 17 β AND
PROGESTERONE ON THE IN VITRO
MATURATION OF FOLLICULAR RAB-
BIT AND COW OOCYTES

Trials involving 539 follicular rabbit oocytes and

306 follicular cow oocytes were conducted to study the effects of estradiol 17β and progesterone on their subsequent maturation in vitro. Rabbit and cow oocytes were removed from their ovarian follicles by the previously discussed method and were incubated for 12 hours and 40 hours, respectively, in TC 199 plus 10% homologous serum for rabbit oocytes or 15% homologous serum for cow oocytes. In addition, the medium was supplemented with 100 IU of penicillin and 100 μ g of streptomycin per ml and buffered with bicarbonate to a pH of 7.0 to 7.2. All oocytes were incubated in a drop-let of medium under mineral oil at 37°C with 5% CO₂ in high humidity air. Since both estradiol 17β and progesterone are insoluble in aqueous solutions, it was necessary to dissolve them in absolute methanol. Stock solutions of progesterone (1 gm per 250 ml absolute methanol) and estradiol 17β (2 gm per 250 ml absolute methanol) were maintained. The methanol was added to the medium to give steroid concentrations of 48 μ g/ml of estradiol 17β or 32 μ g/ml of progesterone. On two of the three subgroups, mechanical agitation was employed to remove the cumulus oophorus cells or to remove all supporting cumulus oophorus and corona radiata cells. In addition to adding estradiol 17β and progesterone to the media, control incubations were conducted

using TC 199 plus serum only or TC 199 plus serum and methanol. The amount of absolute methanol added to the media corresponded to the amount added with the estradiol 17 β and progesterone.

The results of this experiment are summarized in Table 6 (rabbit oocytes) and Table 7 (cow oocytes).

RESULTS OF EXPERIMENT III:

a. Effect of absolute alcohol

Sixty-one follicular rabbit oocytes and 35 follicular cow oocytes were incubated in the TC 199 and serum medium containing, in addition, an amount of absolute methanol equivalent to that added with the estradiol 17 β and progesterone of the other media. Application of a Chi Square analysis to the results indicated that the absolute alcohol in the medium did not significantly alter ($P > .01$) the in vitro maturation behaviour of either the rabbit or the cow oocytes when compared with the control oocytes incubated in the medium containing no alcohol or steroid. This was true for oocytes with both cumulus oophorus and corona radiata cells intact, oocytes with only the corona radiata cells intact, and completely denuded oocytes.

b. Effect of estradiol 17 β

Table 6. Influence of estradiol 17β and progesterone on maturation of rabbit oocytes

	Immature	Partially Mature	Mature
<u>Medium only (control):</u>			
Control (normal) oocytes			
Incubated immediately	8 (5%)	11 (7%)	135 (88%)
Incubation delayed	9 (14%)	14 (23%)	39 (63%)
Cumulus removed	5 (9%)	15 (25%)	35 (66%)
Completely denuded	25 (66%)	6 (16%)	7 (18%)
Cumulus re-added	7 (30%)	5 (22%)	11 (48%)
<u>Medium plus absolute methanol:</u>			
Control (normal) oocytes	1 (4%)	4 (18%)	18 (78%)
Cumulus removed	3 (24%)	5 (38%)	5 (38%)
Completely denuded	12 (48%)	8 (32%)	5 (20%)
<u>Medium plus estradiol 17β:</u>			
Control (normal) oocytes	10 (43%)	11 (47%)	2 (9%)
Cumulus removed	5 (36%)	8 (57%)	1 (7%)
Completely denuded	37 (73%)	6 (12%)	8 (15%)
<u>Medium plus progesterone:</u>			
Control (normal) oocytes	3 (13%)	9 (39%)	11 (48%)
Cumulus removed	0	2 (50%)	2 (50%)
Completely denuded	12 (30%)	18 (44%)	11 (26%)

Table 7. Influence of estradiol 17 β and progesterone on maturation of cow oocytes

	Immature	Partially Mature	Mature
<u>Medium only (control):</u>			
Control (normal) oocytes			
Incubated immediately	4 (4%)	28 (27%)	71 (69%)
Incubation delayed	4 (12%)	9 (26%)	21 (62%)
Cumulus removed	0	4 (36%)	7 (64%)
Completely denuded	27 (75%)	7 (19%)	2 (6%)
Cumulus re-added	3 (43%)	2 (28.5%)	2 (28.5%)
<u>Medium plus absolute methanol:</u>			
Control (normal) oocytes	1 (7%)	3 (21%)	10 (72%)
Cumulus removed	1 (10%)	4 (40%)	5 (50%)
Completely denuded	7 (64%)	3 (27%)	1 (9%)
<u>Medium plus estradiol 17β :</u>			
Control (normal) oocytes	11 (65%)	4 (23%)	2 (12%)
Cumulus removed	7 (78%)	1 (11%)	1 (11%)
Completely denuded	11 (85%)	2 (15%)	0
<u>Medium plus progesterone:</u>			
Control (normal) oocytes	1 (6%)	36 (40%)	8 (54%)
Cumulus removed	0	7 (54%)	6 (46%)
Completely denuded	1 (8%)	6 (46%)	6 (46%)

Estradiol 17β reduced ($P < .01$) the number of rabbit oocytes and reduced ($P < .05$) the number of cow oocytes which matured to the first polar body stage when compared with oocytes incubated in the absence of steroid. This apparent block to complete maturation occurred in both normal follicular oocytes and in oocytes with only the corona radiata cells attached. The influence of the estradiol 17β on the normal oocytes containing both cumulus oophorus and corona radiata cells was not significantly different ($P > .05$) from its influence on oocytes from which the cumulus oophorus cells had been removed. Three (8%) out of 37 non-denuded rabbit oocytes and 3 (11.5%) out of 26 non-denuded cow oocytes matured to extrude the first polar body. Nineteen (51%) non-denuded rabbit oocytes and 8 (31%) non-denuded cow oocytes exhibited germinal vesicle breakdown compared with 249 (92%) non-denuded control rabbit oocytes and 140 (95%) non-denuded control cow oocytes which exhibited germinal vesicle breakdown.

Within the estradiol 17β treatment, maturation beyond the germinal vesicle stage occurred in significantly more ($P < .05$) non-denuded rabbit oocytes (59.4%) than in the completely denuded rabbit oocytes (27%). Of the 59.4%, 51.3% matured only to metaphase of the first meiotic division and 8.1% matured to extrude the first polar body. Of

the 27% of the denuded rabbit oocytes which matured beyond the germinal vesicle stage, 12% matured to metaphase I and 15% matured to extrude the first polar body. Cow oocytes differed in this respect in that the maturation behaviour in vitro of the non-denuded cow oocytes incubated in the presence of estradiol 17β was not different ($P > .05$) from that of completely denuded oocytes also incubated in the presence of estradiol 17β .

The maturation behaviour of the denuded rabbit and cow oocytes incubated in the estradiol 17β medium was not significantly different ($P > .05$) from that of the denuded oocytes incubated in the steroid free, control medium. The incidence of polar body formation in non-denuded rabbit oocytes and the incidence of GVBD and polar body formation in non-denuded cow oocytes incubated in the presence of estradiol 17β was not significantly different from that observed in completely denuded oocytes incubated in the control medium.

c. Effect of progesterone

Within the progesterone treatment, there was no difference ($P > .05$) in the maturation behaviour of normal follicular rabbit oocytes (cumulus and corona radiata intact) and rabbit oocytes from which the cumulus oophorus

cells had been removed. A similar maturation behaviour was observed with both groups of non-denuded cow oocytes. Unlike the maturation behaviour of denuded vs non-denuded rabbit and cow oocytes incubated in the control medium, there was no significant decrease in the incidence of GVBD and polar body formation in denuded rabbit and cow oocytes incubated in the presence of progesterone compared with the observed incidence in non-denuded oocytes incubated in the presence of progesterone.

The incidence of polar body formation (48%) in non-denuded rabbit oocytes was significantly higher ($P < .01$) when incubated in the presence of progesterone than the incidence (8%) observed in non-denuded rabbit oocytes incubated in the presence of estradiol 17β . With non-denuded cow oocytes, the incidences of both GVBD (96%) and polar body formation (50%) were higher ($P < .01$) when oocytes were incubated in the presence of progesterone than when oocytes were incubated in the presence of estradiol 17β (32% GVBD; 12% polar body formation). Significantly more ($P < .05$) denuded rabbit and cow oocytes, incubated in the presence of progesterone, exhibited GVBD than did denuded oocytes incubated in the presence of estradiol 17β .

There was no difference ($P > .05$) between the incidence of GVBD and polar body extrusion in non-denuded

oocytes incubated in the presence of progesterone and that of non-denuded oocytes incubated in the control medium. With completely denuded cow oocytes, the incidence of GVBD and polar body formation was significantly higher ($P < .01$) when oocytes were incubated in the presence of progesterone than when incubated in the control medium. With completely denuded rabbit oocytes, however, the incidence of polar body formation (26%) observed in oocytes incubated in the presence of progesterone was not different ($P > .05$) from the incidence of polar body formation (18%) in the denuded oocytes incubated in the control medium. However, the incidence of GVBD (70%) in such oocytes was higher ($P < .05$) when incubated in the presence of progesterone than when incubated in the control medium (34%).

EXPERIMENT IV: FERTILIZATION IN VIVO OF OOCYTES MATURED IN VITRO

Trial 1: Rabbit Oocytes

Two mature female New Zealand White rabbits were mated naturally during normal estrus. Eight hours after mating, both rabbits were anesthetized with halothane ("Fluothane", Ayerst Laboratories, Montreal, Canada). A ventral midline incision was made beginning at the umbilicus and

extending caudally about 2 inches. Each ovary and oviduct was carefully exteriorized. Five follicular rabbit oocytes, which had been cultured during the previous 12 hours, were transferred into each oviduct. A glass-tipped micropipette was used in the manipulation and transfer of the oocytes. The ovary and oviduct were returned to the peritoneal cavity and the incision closed.

Twenty-six hours after surgery, both recipients were killed. The ovaries, oviducts, and uterus of each rabbit were removed. Ovaries were examined for the presence and number of corpora lutea, indicating the number of ovulated ova which would be present in each oviduct. Each oviduct was flushed from the infundibulum to the utero-tubal junction with 5 ml of physiological saline, then in the opposite direction with 5 ml. With the aid of a stereoscopic microscope (Figure 3) the ova were recovered from the 10 ml of saline.

Microscopic examination of the ova recovered from the first rabbit revealed that 7 of the 8 ova recovered from the left oviduct and 7 of the 9 ova recovered from the right oviduct had cleaved to at least the two-celled stage. The left and right ovaries contained 4 and 5 corpora lutea, respectively, indicating that at least 3 of the 5 ova trans-

ferred into the left oviduct and at least 2 of the 5 ova transferred into the right oviduct had been fertilized.

Microscopic examination of the ova recovered from the second rabbit revealed that all 11 ova recovered from the left oviduct and 7 of the 8 ova recovered from the right oviduct had cleaved to at least the two-celled stage. Six and 4 corpora lutea were found on the left and right ovaries, respectively, indicating that all 5 of ova transferred into the left oviduct and at least 4 of the 5 ova transferred into the right oviduct had been fertilized.

Trial 2: Cow Oocytes

Four heifers were treated with subcutaneous implants of melengestrol acetate (MGA, British Drug Houses, Toronto, Canada) for at least 18 days prior to transferring oocytes. This treatment was found to effectively inhibit estrus in all animals. Normal standing estrus was observed about 3 days following removal of the implant. All animals had exhibited estrus prior to the hormone treatment.

Heifer No. 1: Attempts to artificially inseminate heifer No. 1 during estrus failed because of a very small cervix. The animal was anesthetized by standard lumbar epidural analgesia procedures. A high lumbar incision, 12 to 16 inches in length, was made on the heifer's right side.

Because of the unsuccessful attempts to artificially inseminate the animal, surgical insemination was carried out with semen contents of two standard glass frozen semen vials being injected directly into the uterine body. Twenty follicular cow oocytes, which had been recovered from the abattoir and cultured for 40 hours, were transferred into the right oviduct (Figure 22).

Difficulty was encountered in positioning the oviduct for the transfer since the reproductive tract of the heifer was small. Excessive handling of the reproductive tract resulted.

The heifer was killed 3 days after the transfer. The uterus, oviducts, and ovaries were removed. One corpus luteum was observed on the right ovary. The oviduct and the uterus were carefully flushed with physiological saline. Only one ovum was recovered and microscopic examination revealed that it was not fertilized. No spermatozoa were found on the ovum or in the reproductive tract.

Heifer No. 2: The heifer was prepared for the transfer in a manner similar to that described for heifer No. 1. Ten follicular oocytes, which had been cultured for 36 hours, were transferred into the right oviduct 8 hours after artificially inseminating the animal with 3 ml of freshly

ejaculated semen. A sample of the semen was examined microscopically to ensure adequate spermatozoan viability.

The animal was killed two days after the transfer. Flushing of the oviduct resulted in the recovery of 6 ova. Microscopic examination of these ova revealed that 4 had undergone abnormal cleavage or segmentation. None of the ova was fertilized. No spermatozoa were found on the ova or in the reproductive tract.

Heifers No. 3 and No. 4: The two animals were artificially inseminated with 3 ml of freshly ejaculated semen. Both heifers were anesthetized by standard caudal epidural analgesia procedures. A vaginal speculum, to which 2 battery operated lights had been attached (Figure 27), was inserted into the vagina. A five inch incision was made through the dorsal portion of the vagina. Manually, each ovary and oviduct was exteriorized from the peritoneal cavity through the vaginal incision. Because of the small size of the reproductive tract of heifer No. 3, attempts to position the oviduct closer to the vulva proved unsuccessful and no oocytes were transferred. Similar problems were encountered with heifer No. 4 in that the small size of the vulva, vagina, and uterus prevented the exposure of the oviduct necessary for transfer of oocytes.

Trial 3: Cow Oocytes Transferred into Sheep

Four mature ewes which had failed to conceive during the normal breeding season were used in this trial. The ewes had been kept under controlled light conditions, the amount of light at the time of the experiment being similar to that of the normal breeding season.

(a) Ewes No. 1 and No. 2 were treated with a subcutaneous injection of 750 I.U. of pregnant mares' serum (PMS, "Equinex", Ayerst Laboratories, Montreal, Canada) followed, 48 hours later, by an intramuscular injection of 500 I.U. of human chorionic gonadotrophin (HCG, "A.P.L.", Ayerst Laboratories, Montreal, Canada). Both ewes were artificially inseminated 20 hours after the injection of HCG.

Seven hours post-insemination, the ewes were anesthetized with halothane. A mid-ventral incision was made, beginning at the umbilicus and extending caudally 5 inches. Follicular cow oocytes which had been recovered from the abattoir and cultured for 38 hours were used in the transfers. Five oocytes were transferred into each oviduct of ewe No. 1 and 4 oocytes were transferred into each oviduct of ewe No. 2.

Ewe No. 1 was killed 40 hours after the transfer. One corpus luteum was observed on the left ovary. No ova

were found in the left oviduct. One 1-celled ovum was found in the left uterine horn. The right ovary contained two corpora lutea. Three cow ova and 2 sheep ova were recovered from the right oviduct. All ova were one-celled and the cytoplasm of the cow ova had shrunken (Figure 26). No spermatozoa were found on the ova or in the reproductive tract.

Ewe No. 2 was killed 60 hours after the transfer. No corpora lutea were found on the left ovary. Two ova, both one-celled were recovered from the left oviduct. No corpora lutea were observed on the right ovary. Four ova were recovered from the right oviduct. The cytoplasm of all ova recovered had shrunken as with the cow ova recovered from ewe No. 1. No spermatozoa were found on the ova or in the reproductive tract.

(b) Vaginal pessaries containing flurogestone acetate ("Cronolone", G.D. Searle, Chicago, U.S.A.) were inserted into the vaginas of ewes No. 3 and No. 4, 95 hours after the injection of HCG (as described in part (a) of this trial). The pessary in ewe No. 3 was lost during the 20 day treatment period. The pessary in ewe No. 4 was removed at this time. Similar treatment with PMS and HCG was commenced 48 hours following the removal of the pessary from

ewe No. 4. Eight hours after artificially inseminating the animals, 5 follicular cow oocytes were transferred into each oviduct of ewe No. 3 and ewe No. 4.

Both animals were killed 40 hours after the transfer. No corpora lutea were found on the ovaries of ewe No. 3. Two one-celled ova were recovered from the left oviduct. Shrinking of the cytoplasm had occurred. No ova were found in the right oviduct or in the uterus. No spermatozoa were found on the ova or in the reproductive tract.

Two corpora lutea were found on the left ovary and one corpus luteum was found on the right ovary of ewe No. 4. Three ova, all unfertilized, were recovered from each oviduct. Cytoplasmic shrinking was observed in the cow oocytes. No spermatozoa were found.

EXPERIMENT V: CLEAVAGE IN VITRO OF OVA
FERTILIZED IN VIVO

New Zealand White does, weighing from 3.5 to 4.5 kg were given 6 subcutaneous injections of 50 I.U. of PMS each, administered at 12 hour intervals. Twelve hours following the last injection, an ovulatory dose (100 I.U.) of HCG was administered, intravenously. The animals were then mated naturally. Approximately 24-26 hours following the injection

of HCG, the animals were killed and the oviducts immediately excised. Ten ml of warm (37°C) culture medium was used to flush each oviduct. Two-celled ova recovered from the flushings were quickly incubated in a droplet of medium under mineral oil in a humidified mixture of 5% CO₂:95% air at 37°C. Ova were cultured for at least 24 hours, at which time they were observed microscopically for indications of cleavage beyond the two-celled stage.

Trials involving 283 two-celled rabbit ova were conducted. Sixty-eight ova were incubated in 100% rabbit serum, 73 ova were incubated in TC 199 plus 10% rabbit serum, 47 ova were incubated in TC 199 plus 50% rabbit serum, and 95 ova were incubated in 100% pig serum. All media were supplemented with 100 I.U. of penicillin and 100 µg of streptomycin per ml.

Fifty-four (86%) of the ova cultured in 100% rabbit serum cleaved beyond the initial two-celled stage. Twenty-two (30%) of the ova cultured in TC 199 plus 10% rabbit serum developed beyond the two-celled stage, whereas, 20 (42%) of the ova cultured in TC 199 plus 50% serum cleaved to at least the 4-celled stage. Thirty-five (37%) of the ova cultured in 100% pig serum cleaved beyond the two-celled stage.

Application of a Chi Squared analysis to the above results indicated that more ova ($P < .05$) underwent cleavage beyond the two-celled stage when cultured in 100% rabbit serum than when culture in the three other media. The maximum cleavage stage observed with ova cultured in 100% pig serum was the 8-celled stage (Figure 28) whereas the maximum cleavage stage observed with ova culture in 100% rabbit serum was the 12-celled stage (Figure 29).

DISCUSSION

The fact that follicular oocytes will resume meiosis and mature to metaphase of the second meiotic division in vitro has been well documented and demonstrated in several mammalian species including the mouse, rabbit, cow, pig, rhesus monkey and human. Various media have successfully been used to incubate oocytes. The culture medium consisting of TC 199 and homologous serum was selected on the basis of the results obtained by Edwards (1965b). During the present experiments, this medium was observed to be reliable for the culture of follicular cow and rabbit oocytes. The fact that very few follicular pig oocytes matured in vitro in the TC 199 medium differs from the observations of Edwards (1965b). The difference, however, may not be due to the medium used. Whereas all rabbit and cow oocytes were recovered from sexually mature, cycling animals, all pig oocytes were recovered from prepuberal gilts. Further trials involving oocytes from both immature and mature animals would indicate if sexual maturity is a necessary requirement for maturation of pig oocytes. The observations of Onuma and Foote (1969), that 9-17 week old calves could be superovulated and inseminated, resulting in

fertilized ova, indicate that in the bovine, at least, sexual maturity is not necessary or, if it is, superovulatory procedures overcome the immature condition of the ovary.

The value of pre-exposing the culture medium to the 5% CO₂ of the incubator was demonstrated. Incubation of oocytes in non-pre-exposed medium significantly reduced the number of oocytes maturing to the first polar body. Since the non-pre-exposed medium had been heated to 37°C prior to incubation, influences imposed upon oocyte maturation can be attributed to differences in pH of the media. The non-pre-exposed medium had an initial pH of 8.5, whereas, the pH of the pre-exposed medium at the time of incubation was 7.0-7.2. Further examination of the results reveal that the decrease in the number of polar bodies extruded in the non-pre-exposed medium corresponded with an increase in the number of oocytes which remained in the germinal vesicle stage. This indicates that the initially high pH of the non-pre-exposed medium had a detrimental effect on the oocytes resulting in more oocytes remaining in the germinal vesicle stage.

No differences in maturation were observed when oocytes were incubated under mineral oil or when they were incubated in dishes containing the medium only. However, it

was decided that for future trials, mineral oil would be used because of the improved convenience in isolating and locating individual oocytes.

Although oocytes were first matured in vitro several years ago (Pincus and Enzmann, 1935), most of the work concerning the culture of oocytes has been reported during the past 10 years. Much emphasis in these studies has been placed on improving techniques, developing new media, and maturing the follicular oocytes with the view of fertilizing them in vivo or in vitro. Few reports have attempted to elucidate the factors involved in stimulating maturation in vivo or in vitro. The mechanism by which luteinizing hormone induces oocyte maturation and ovulation in vivo is not known. The fact that maturation in vitro occurs in the absence of LH suggests that LH serves as a mechanism to trigger changes within the follicle which, in turn, results in maturation of the oocyte. Similar changes could result by puncturing the follicle and removing the oocyte.

The inhibition of maturation that was observed following complete oocyte denudation indicates that the supporting cumulus oophorus and corona radiata cells exhibit an important influence on the resumption of maturation of follicular oocytes. The cumulus oophorus cells do not appear to be necessary since oocytes denuded of these cells, leaving the

corona radiata intact, matured as did the normal control oocytes. Removal of the corona radiata cells significantly inhibited oocyte maturation. This might indicate that agitation employed in the denudation process may have had a detrimental effect on the oocytes. However, re-addition of cumulus oophorus cells (Figures 21, 22 and 23) to the drop-lets of medium containing denuded oocytes did not result in a significant decrease in polar body extrusion, demonstrating that agitation did not result in the observed decrease in maturation and also that cumulus oophorus cells alone, without corona radiata, were capable of supporting maturation. Such observations demonstrate that the supporting cumulus oophorus and corona radiata cells are directly involved in the induction of oocyte maturation. A similar decrease in human oocyte maturation following denudation was observed by Kennedy and Donahue (1969).

The mechanism by which the supporting cells induce oocyte maturation is not known. It is known, however, that processes from the corona radiata cells (Figure 5) pass through the zona pellucida of the oocyte and it is possible that these tube-like processes may be involved in the relationship between the corona radiata cells and oocyte maturation.

Studies with frog (Rana pipiens) oocytes have implicated a relationship between the supporting cells surrounding the oocyte and the ovarian hormone, progesterone, since denuded frog oocytes failed to mature in vitro unless progesterone was added to the medium (Schuetz, 1967a,b; Masui, 1967). There is a possibility that a similar relationship may exist with mammalian oocytes and it was decided to study the effects of the ovarian hormones progesterone and estradiol 17 β on maturation of rabbit and cow oocytes.

Since both progesterone and estradiol 17 β are highly insoluble in aqueous solutions, it was necessary to dissolve them in absolute alcohol prior to adding them to the TC 199 medium. Absolute methanol was selected as the alcohol with the anticipation that it would not serve as an additional energy source. The absolute methanol did not have an adverse effect on the oocytes indicating that for such studies it was a suitable solvent for the steroids.

The observed effects of progesterone and estradiol 17 β on the maturation of follicular rabbit and cow oocytes are interesting. Of these effects, the most important are the inhibition of polar body formation in non-denuded oocytes incubated in the presence of estradiol 17 β and the increased incidence of germinal vesicle breakdown and polar body

formation in denuded oocytes incubated in the presence of progesterone (Figure 20). Estradiol 17β appeared to exert its inhibiting influence on the maturation of the rabbit oocytes following the initiation of germinal vesicle breakdown, although prior to the time of polar body extrusion. With cow oocytes, the estradiol 17β exerted its inhibitory influence prior to the time of germinal vesicle breakdown. This difference between rabbit and cow oocytes might possibly be explained by the difference in maturation times of the oocytes. If more than 4 or 5 hours is required for estradiol 17β , at the level and conditions used, to completely exert its influence, the rabbit oocytes would have already proceeded beyond the germinal vesicle stage, whereas, the cow oocytes would not have. The same situation might possibly explain the fact that incubating denuded oocytes in the presence of progesterone increased both germinal vesicle breakdown and polar body extrusion in cow oocytes and only germinal vesicle breakdown in rabbit oocytes. If progesterone requires a period of a few hours to exert its stimulating influence on oocyte maturation, the 12 hour incubation period for rabbit oocytes may have been insufficient for complete realization of the influence of progesterone on denuded rabbit oocytes. A longer incubation period for

such oocytes would have indicated if this was the case or if the differences were due to faulty procedures at the time of the incubations.

The mechanism by which estradiol 17β and progesterone exert their influence on oocytes has not been reported. Reports concerning the induction of oocyte maturation are relatively few and present attempts to explain possible mechanisms by which follicular oocytes begin to mature in vivo or in vitro following long periods of dormancy necessitate combining observations obtained from several species. The present observations with rabbit and cow oocyte denudation and the addition of progesterone and estradiol 17β to the culture medium indicate a similar behaviour in vitro of rabbit, cow, and amphibian oocytes. Although the reproductive physiology of amphibians is quite different in many respects from that of mammals, it appears that factors involved in inducing germinal vesicle breakdown and polar body extrusion in mammals and amphibians may be similar.

A possible relationship between the observed influence of the granulosa cells and the observed influence of progesterone lies in the observations of Channing (1966) that equine granulosa cells were capable of synthesizing progesterone when incubated in vitro. Removal of the granulosa cells from

the follicle appeared to provide the stimulus for luteinization of the cells. Such luteinization of the granulosa cells may have served as a source of the increased progesterone levels within the ovarian follicles immediately following LH stimulation in the rabbit (Hilliard et al., 1961, 1963; Solod et al., 1966). Until LH stimulation in vivo or incubation of the oocyte in vitro, the Graafian follicle is under the influence of estrogen. Chatterton et al. (1969) observed decreased estrogen synthesis by the ovaries of rats following LH stimulation.

On the basis of such reports and of the observations provided by the present experiments, it is proposed that the ovarian hormone progesterone provides the stimulus for the resumption of meiosis in vivo and in vitro, leading to maturation of the oocyte. Estrogen, on the other hand, may function to inhibit maturation, the inhibitory influence being overcome following LH stimulation of the follicle or removal of the oocyte from the inhibitory influence. Such an inhibitory and stimulatory mechanism of estrogen and progesterone would not only explain observations provided by incubation of normal oocytes, denuded oocytes, and denuded oocytes in the presence of re-added granulosa cells, but, would also help to explain follicular atresia, the occurrence of which

increases following sexual maturity. It may be possible that the stimulating dose of LH on a given follicle may be sufficient to induce luteinization of the granulosa cells yet not sufficient to result in ovulation of the oocyte from the follicle. The increased incidence of atresia in rat oocytes following the administration of progesterone (Young, 1961) could similarly be accounted for. Thus the results of the experiments reported in this thesis and reports by several workers indicate a role of progesterone in the induction of oocyte maturation with estrogen functioning to inhibit maturation of the oocyte within the developing follicle until the time of LH release or removal of the oocyte from the follicle, both of which result in an increase in the production of progesterone.

It was observed that oocytes, incubated within a completely intact follicle, failed to mature beyond the germinal vesicle stage. This failure to mature was not altered even if these intact follicles were incubated in the presence of progesterone. Such observations could be explained by the possible barrier of the follicular membrane to the flow of progesterone. Since luteinization of the cumulus and corona cells is induced by removing them from the follicle, there would be no progesterone production within such

follicles, and the oocytes would remain under the inhibitory influence of estrogens within the follicle.

Tepperman and Tepperman (1960) reviewed several effects of hormones on cells and cell constituents. Relatively little appears to be known concerning the precise action and site of action of hormones on cells. Some hormones are known to cause changes in cell membrane permeability. Various hormones have been shown to effect the transport of amino acids across cell membranes. This particular role may be of significance concerning the influences of progesterone and estradiol 17β on the oocyte. Noall et al. (1957) reported that estradiol caused a marked accumulation of a test amino acid in the cells of the uterus but not in the liver cells, demonstrating a variable effect of a hormone, depending on the tissue. The site of action of the progesterone and the estradiol 17β could be at the surface of the oocyte, the progesterone functioning to permit entry into the oocyte of substances such as amino acids which may be required in the medium. The use of labelled amino acids would facilitate such studies involving maturation of oocytes. Amino acids may not be involved. Biggers et al. (1967) demonstrated that follicular mouse oocytes would mature in vitro when cultured with granulosa cells or when cultured without the cells

but in the presence of pyruvate or oxaloacetate. Donahue and Stern (1968) injected pro-estrus mice with HCG. Granulosa cells and oocytes recovered 30 minutes after the injections were incubated in vitro and were found to produce pyruvate. However, the HCG stimulus did not alter the amount of pyruvate produced in vitro by the granulosa cells. These studies indicate that pyruvate or a similar compound involved in the tricarboxylic cycle may be required in the medium. Progesterone and estradiol 17β may alter permeability of the oocyte membrane to such a compound.

Smith and Ecker (1969) demonstrated that progesterone was able to induce events associated with frog oocyte maturation such as protein synthesis and breakdown of the cortical granules, even if the germinal vesicle of the oocyte was removed. After receiving injections of non-hormone treated germinal vesicle material, such enucleated oocytes cleaved normally in response to transplanted nuclei. Smith and Ecker suggested that, in frog oocytes, progesterone acts at an extracellular site, such as the surface of the cell, to induce maturation. Injection of progesterone into the cell did not induce maturation.

The experiments reported in this thesis demonstrate that progesterone and estradiol 17β have a role in stimulating and inhibiting maturation of rabbit and cow oocytes.

However, they are not sufficient to demonstrate the site and mechanism of action of the hormones. The use of both hormones in the same medium at various concentration ratios would have indicated if the action of progesterone opposes that of estradiol 17β . Exposing the oocytes to the progesterone for a short period of time and the use of media designed to provide only the proper osmotic pressure rather than to serve as a nutrient source, would indicate if the oocyte depends on such an added source of nutrients for maturation and if progesterone functions at the surface of the cell.

Although attempts to transfer and fertilize cow oocytes did not prove successful, it is unlikely that the culture techniques involved in maturing oocytes in vitro seriously affected their fertilizability. Fertilized, two-celled rabbit ova were recovered following transfer of cultured oocytes into a recipient female. Only a small percentage of the cultured oocytes appeared to be degenerate (Figures 24 and 25) following culture and it is possible that such oocytes were in a degenerate condition prior to incubation. Difficulties encountered with the transfer of cow oocytes arose mainly from problems in the manipulation of the small reproductive tracts of the heifers. Transfer

of oocytes via laparotomy is an involved and time consuming procedure, the practical importance of which is very limited. Transfer of unfertilized oocytes via vaginal incision appears to be feasible only with mature animals with larger reproductive tracts. Such a method would be of practical importance because of the relative simplicity and short time involved. Anesthesia and surgery could be done in about 15 minutes. The fact that surgery is required emphasizes the value of suitable non-surgical ova transfer techniques involving the transfer of fertilized, cleaving ova. The culture of fertilized ova in vitro has been well demonstrated. The present experiments indicate that homologous serum would be a suitable culture medium for cleavage in vitro of fertilized ova.

A problem with the use of follicular oocytes in non-surgical transfers arises with fertilization of the oocytes. In vitro fertilization of such oocytes would greatly enhance their usefulness. To date, there have been no reports of successful in vitro fertilization of follicular oocytes matured in vitro. The use of laboratory animals such as the rabbit or animals such as the sheep to fertilize cow oocytes in vivo would be of value until suitable in vitro techniques are developed. Attempts by Coggins (1968) to fertilize

foreign gametes in the reproductive tract of the female rabbit and present attempts to fertilize cow oocytes in the sheep oviduct did not prove successful. It appears that the use of different species presents several difficulties. More research is necessary before sufficient evidence is available to determine if oocytes can or cannot be fertilized in the reproductive tracts of other species.

Further uses of methods by which follicular oocytes are matured in vitro are by no means dependent on the fertilization of such oocytes. Such techniques will enable workers to observe the development and changes that take place within the oocyte during ovarian and follicular growth leading to ovulation of the oocyte.

In summary, it appears that the culture media and techniques employed throughout the present experiments provided the necessary requirements for the maturation of follicular rabbit and cow oocytes. The supporting cumulus oophorus and corona radiata cells serve an important role in oocyte maturation. Oocyte maturation was inhibited by removing these cells or by incubating oocytes in the presence of the ovarian hormone estradiol 17β . The inhibition was overcome by re-adding cumulus cells or by incubating the oocytes in

the presence of progesterone. On the basis of these observations and observations reported by several workers, it is concluded that the supporting cells induce oocyte maturation by their production of progesterone. The stimulus for the production of progesterone results from luteinizing hormone or from the removal of the oocyte and supporting cells from the follicle. Until this time, the oocyte is under the inhibitory influence of estrogens within the follicle. The site and mechanism of action of progesterone and estradiol 17 β is not known, although it may be on the surface of the oocyte. Fertilization in vivo of rabbit oocytes cultured in vitro was achieved indicating that such culture procedures do not alter oocyte fertilizability. Difficulties encountered with attempts to transfer and fertilize cow oocytes were primarily results of small reproductive tracts. Further work is necessary to improve the techniques involved with such transfers. Once fertilization of the oocyte has been achieved, suitable media and methods are available by which development of the early embryo in vitro can be carried out.

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APPENDIX

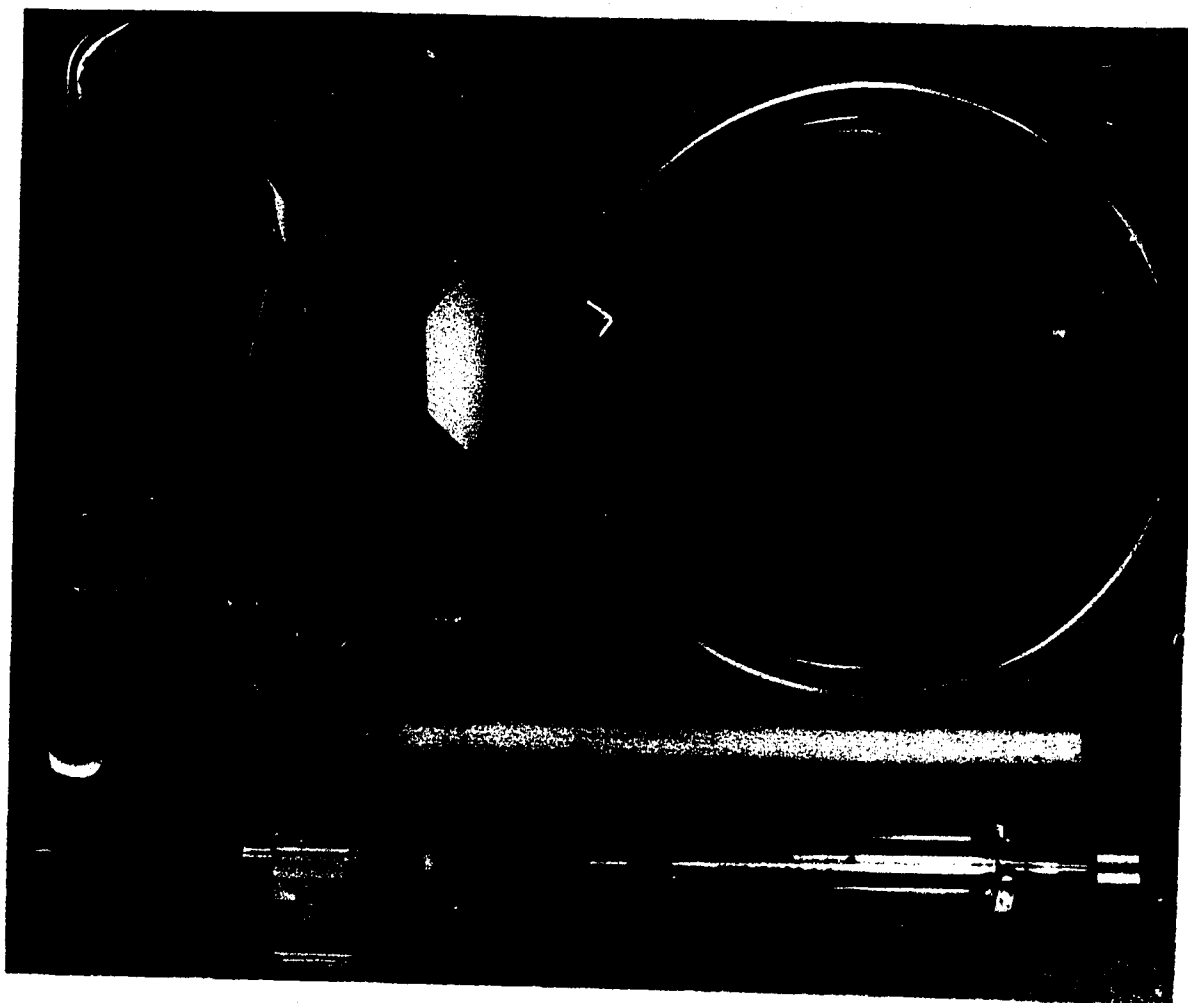


Figure 1. Dissecting equipment used in the recovery of follicular oocytes

- A - Forceps (sharp, curved)
- B - Dissecting scissors (sharp, curved) for cutting follicles
- C - Glass vial for transporting oocytes
- D - Petri dish
- E - Sharp pick for puncturing follicles
- F - Microsyringe for manipulating oocytes

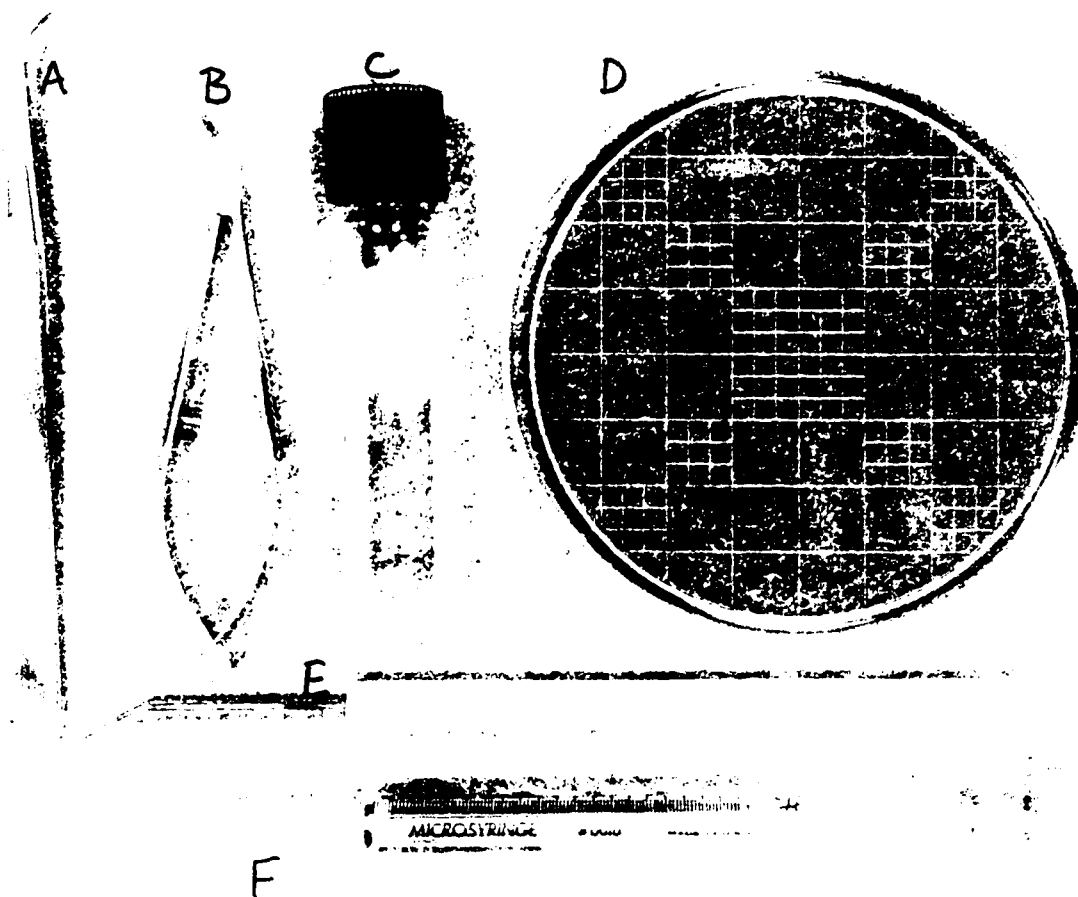
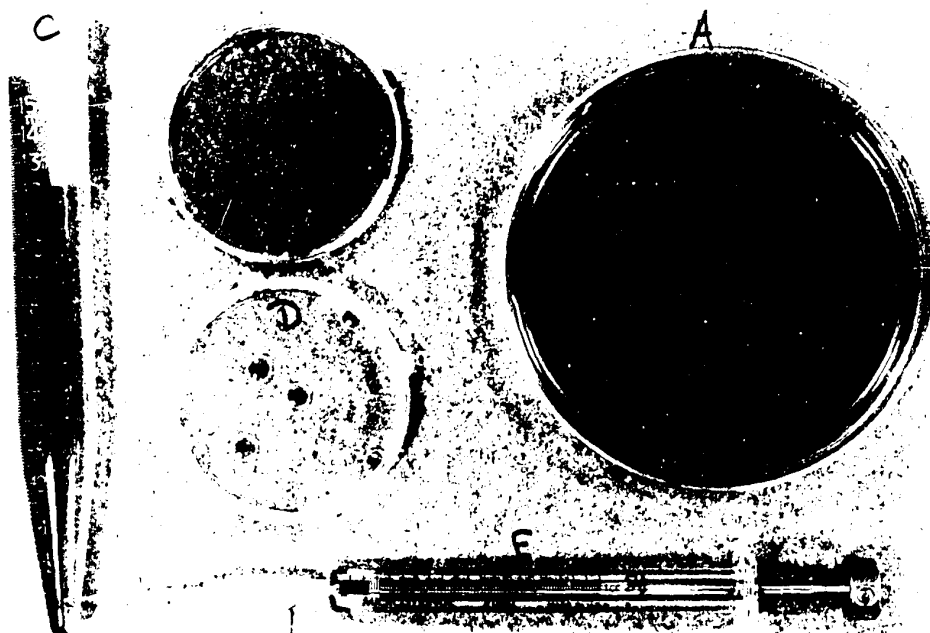




Figure 2. Equipment used in the manipulation and incubation of follicular oocytes.

- A Petri dish (containing culture medium)
- B Culture dish (containing culture medium)
- C Tubes used in agitation of oocytes (denudation)
- D Culture dish containing mineral oil and medium droplets
- E Microsyringe for manipulating oocytes



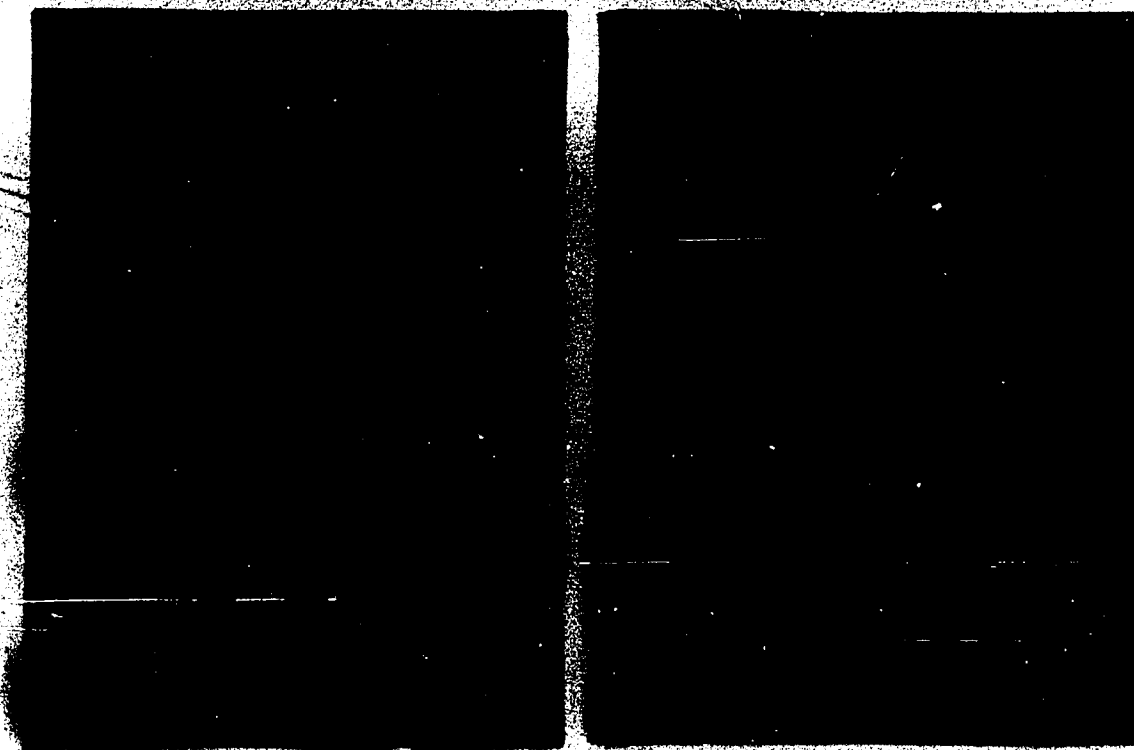


Figure 3. Recovery of oocytes (left) and incubation
in Falcon plastic dishes containing
mineral oil (right).





Figure 4 Phase-contrast photomicrograph of a follicular rabbit oocyte surrounded by corona radiata and cumulus oophorus cells - unstained X550.

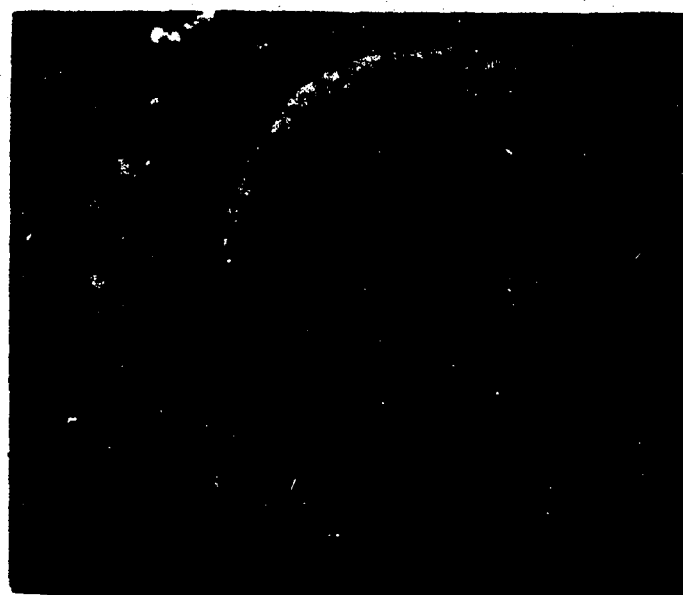


Figure 5 Phase-contrast photomicrograph of a partially denuded rabbit oocyte showing processes from surrounding cells passing through zona pellucida - unstained X500.



Figure 4 Phase-contrast photomicrograph of a follicular rabbit oocyte surrounded by corona radiata and cumulus oophorus cells - unstained X550.



Figure 5 Phase-contrast photomicrograph of a partially denuded rabbit oocyte showing processes from surrounding cells passing through zona pellucida - unstained X500.

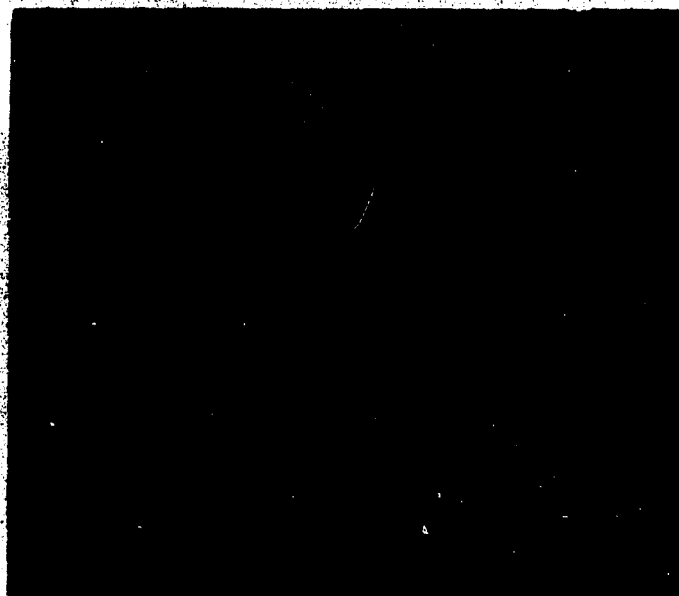


Figure 6 Phase-contrast photomicrograph of a follicular rabbit oocyte as observed immediately after removal from follicle - unstained 550X.



Figure 7 Phase-contrast photomicrograph of a follicular rabbit oocyte after 1 hour of culture showing germinal vesicle - after fixing and staining 700X.

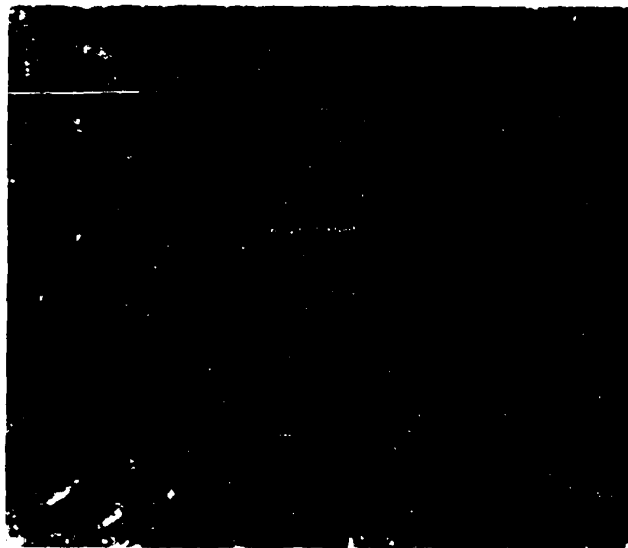


Figure 6 Phase-contrast photomicrograph of a follicular rabbit oocyte as observed immediately after removal from follicle - unstained 550X.



Figure 7 Phase-contrast photomicrograph of a follicular rabbit oocyte after 1 hour of culture showing germinal vesicle - after fixing and staining 700X.



Figure 8 Phase-contrast photomicrograph of a follicular rabbit oocyte after 2 hours of culture showing germinal vesicle - after fixing and staining 450X.



Figure 9 Phase-contrast photomicrograph of a follicular rabbit oocyte after 3 hours of culture showing breakdown of germinal vesicle membrane - after fixing and staining 650X.

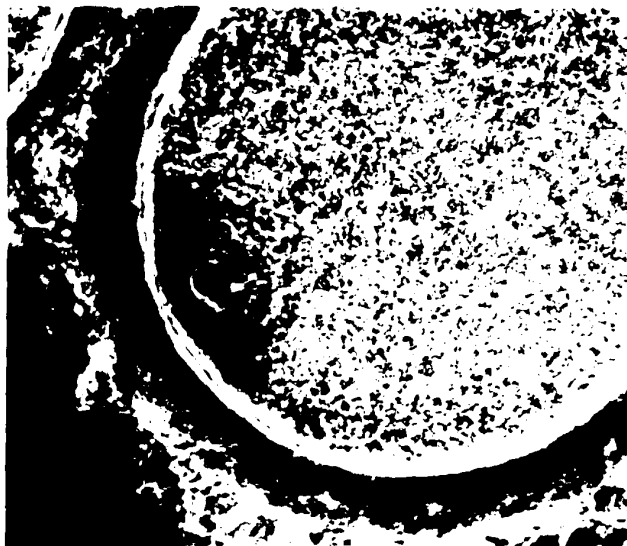


Figure 8 Phase-contrast photomicrograph of a follicular rabbit oocyte after 2 hours of culture showing germinal vesicle - after fixing and staining 450X.

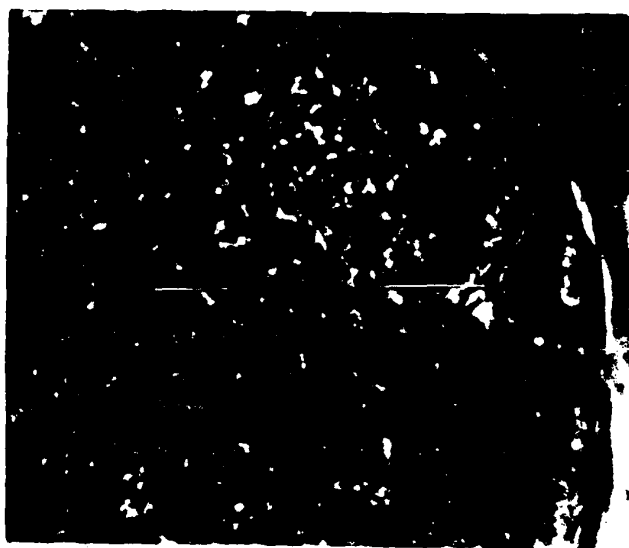


Figure 9 Phase-contrast photomicrograph of a follicular rabbit oocyte after 3 hours of culture showing breakdown of germinal vesicle membrane - after fixing and staining 650X.



Figure 10 Phase-contrast photomicrograph of a follicular rabbit oocyte after 4 hours of culture showing nucleolus during the breakdown of the germinal vesicle - after fixing and staining 750X.

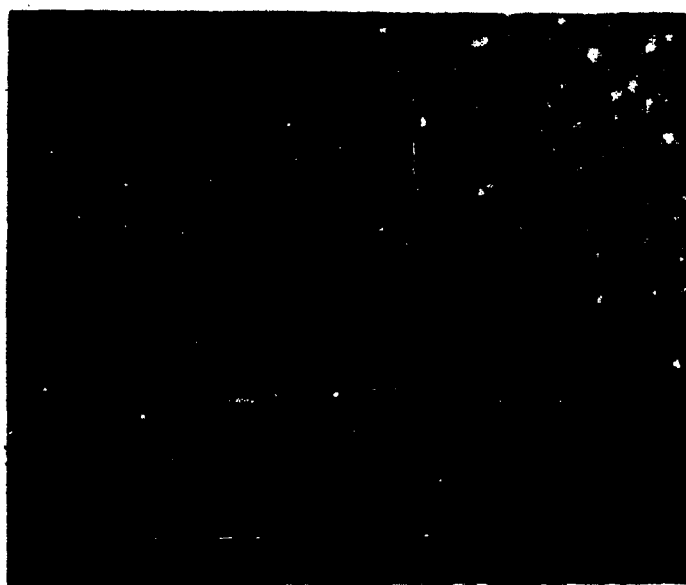


Figure 11 Phase-contrast photomicrograph of a follicular rabbit oocyte after 5 hours of culture showing chromatin and loss of membrane (late germinal vesicle breakdown) - after fixing and staining 650X.

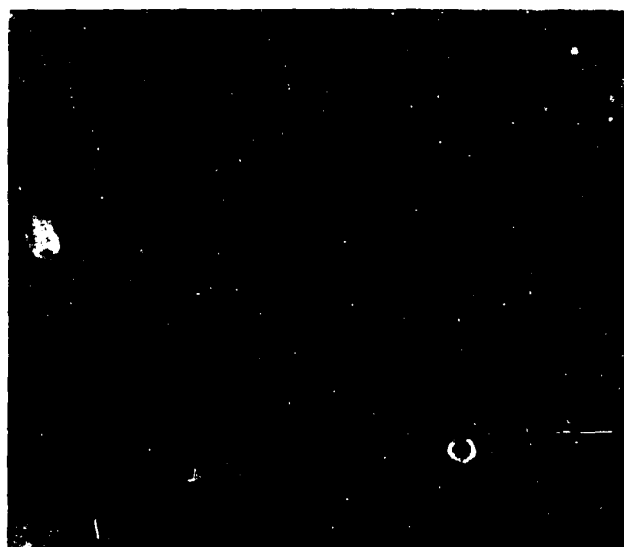


Figure 10 Phase-contrast photomicrograph of a follicular rabbit oocyte after 4 hours of culture showing nucleolus during the breakdown of the germinal vesicle - after fixing and staining 750X.



Figure 11 Phase-contrast photomicrograph of a follicular rabbit oocyte after 5 hours of culture showing chromatin and loss of membrane (late germinal vesicle breakdown) - after fixing and staining 650X.



Figure 12 Phase-contrast photomicrograph of a follicular rabbit oocyte after 6 hours of culture showing early first metaphase (diakinesis) - after fixing and staining 650X.

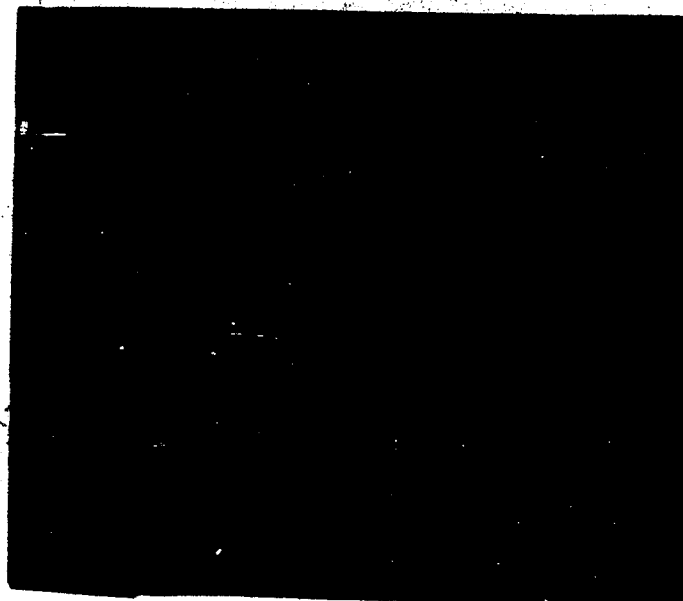


Figure 13 Phase-contrast photomicrograph of a follicular rabbit oocyte after 7 hours of culture showing metaphase plate of the first meiotic division - after fixing and staining 700X.

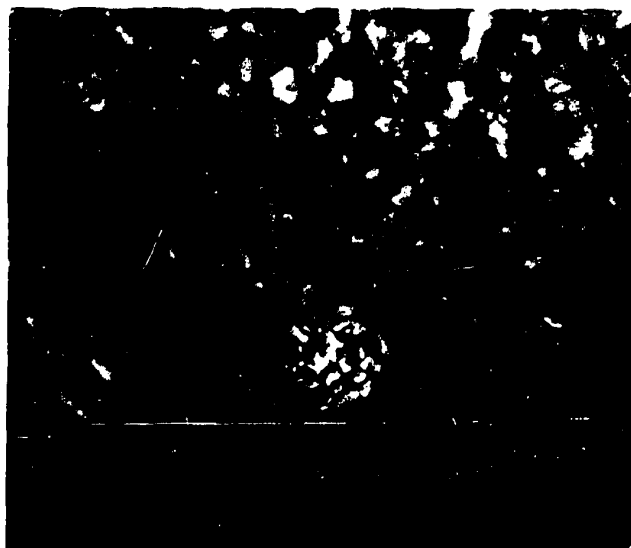


Figure 12 Phase-contrast photomicrograph of a follicular rabbit oocyte after 6 hours of culture showing early first metaphase (diakinesis) - after fixing and staining 650X.



Figure 13 Phase-contrast photomicrograph of a follicular rabbit oocyte after 7 hours of culture showing metaphase plate of the first meiotic division - after fixing and staining 700X.



Figure 14 Phase-contrast photomicrograph of a follicular rabbit oocyte after 8 hours of culture showing early anaphase - after fixing and staining 600X.



Figure 15 Phase-contrast photomicrograph of a follicular rabbit oocyte after 9 hours of culture showing late anaphase - after fixing and staining 550X.

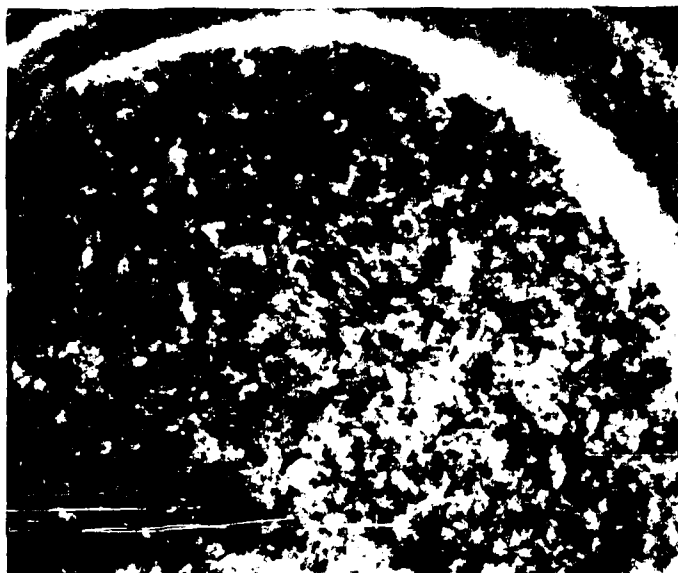


Figure 14 Phase-contrast photomicrograph of a follicular rabbit oocyte after 8 hours of culture showing early anaphase - after fixing and staining 600X.

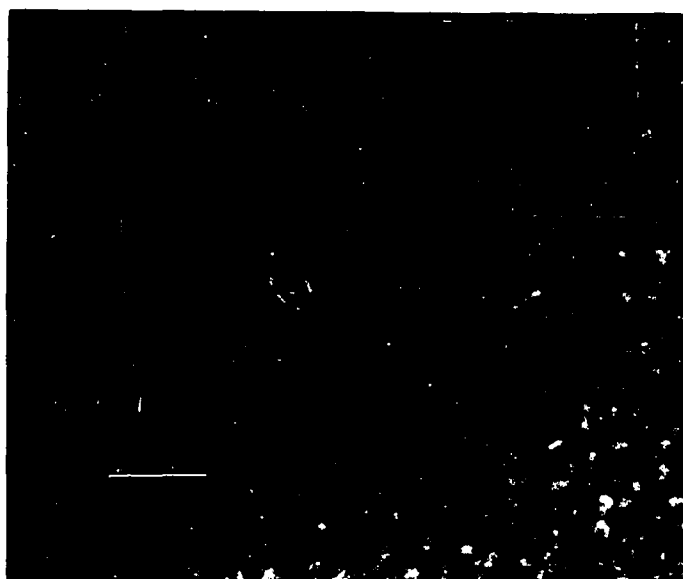


Figure 15 Phase-contrast photomicrograph of a follicular rabbit oocyte after 9 hours of culture showing late anaphase - after fixing and staining 550X.

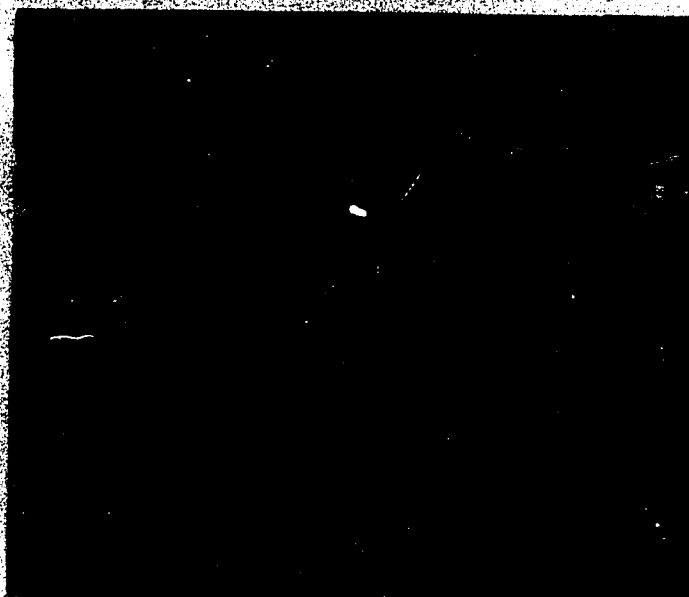


Figure 16 Phase-contrast photomicrograph of a follicular rabbit oocyte after 10 hours of culture showing formation of the first polar body (early telophase) - after fixing and staining 700X.

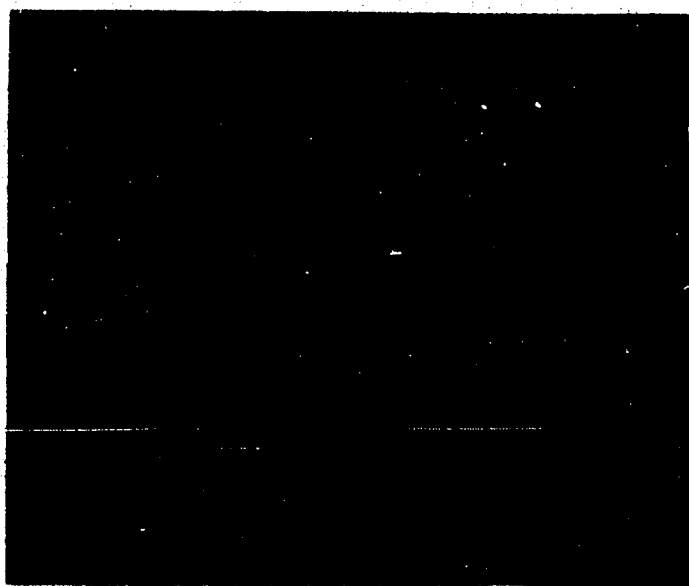


Figure 17 Phase-contrast photomicrograph of a follicular rabbit oocyte after 11 hours culture showing formation of the first polar body (telophase) - after fixing and staining 450X.

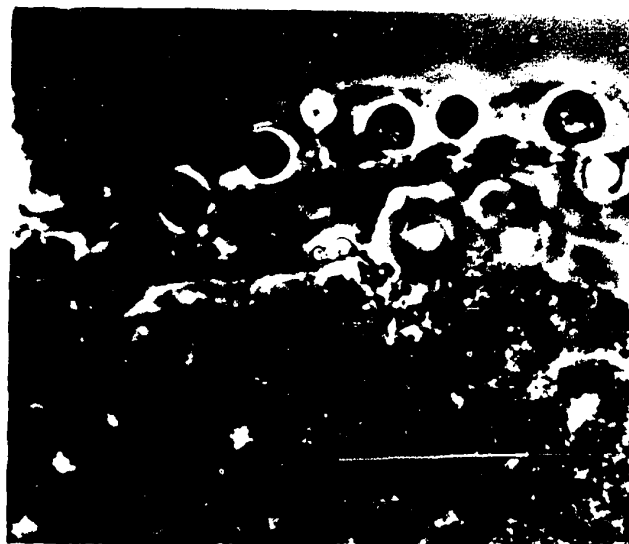


Figure 16 Phase-contrast photomicrograph of a follicular rabbit oocyte after 10 hours of culture showing formation of the first polar body (early telophase) - after fixing and staining 700X.



Figure 17 Phase-contrast photomicrograph of a follicular rabbit oocyte after 11 hours culture showing formation of the first polar body (telophase) - after fixing and staining 450X.



Figure 18 Phase-contrast photomicrograph of a follicular rabbit oocyte after 12 hours of culture showing first polar body formation (telophase) - after fixing and staining 700X.

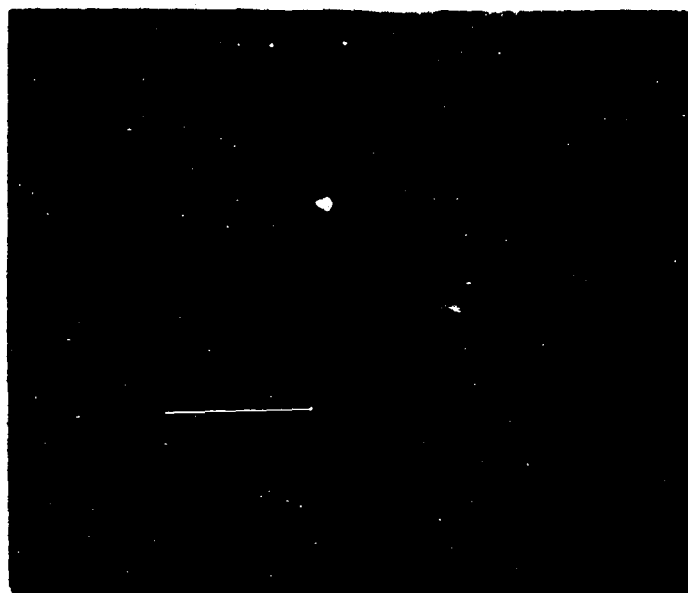


Figure 19 Phase-contrast photomicrograph of a follicular rabbit oocyte after 13 hours of culture showing chromatin of first polar body and second metaphase - after fixing and staining 700X.



Figure 18 Phase-contrast photomicrograph of a follicular rabbit oocyte after 12 hours of culture showing first polar body formation (telophase) - after fixing and staining 700X.

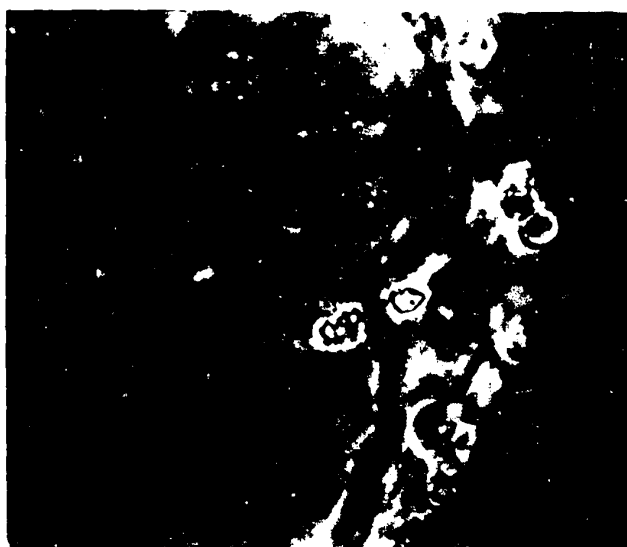


Figure 19 Phase-contrast photomicrograph of a follicular rabbit oocyte after 13 hours of culture showing chromatin of first polar body and second metaphase - after fixing and staining 700X.

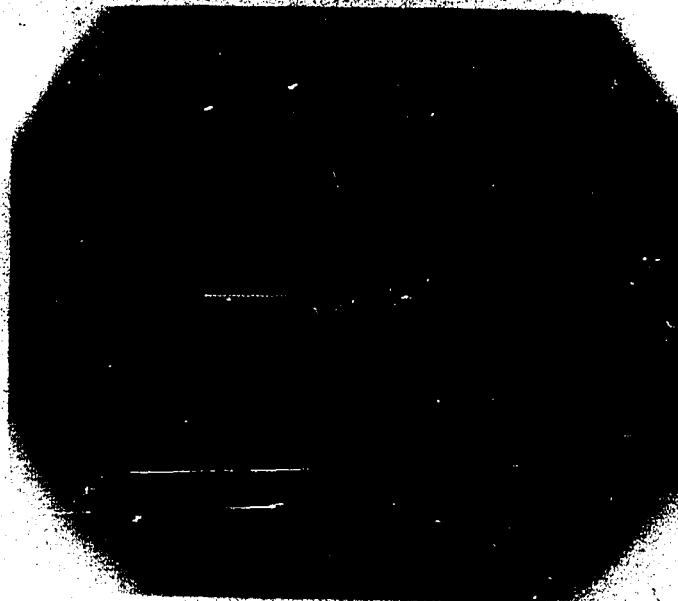


Figure 20 Phase-contrast photomicrograph of a denuded follicular rabbit oocyte incubated in the presence of progesterone - showing first polar body - before fixing and staining 400X.

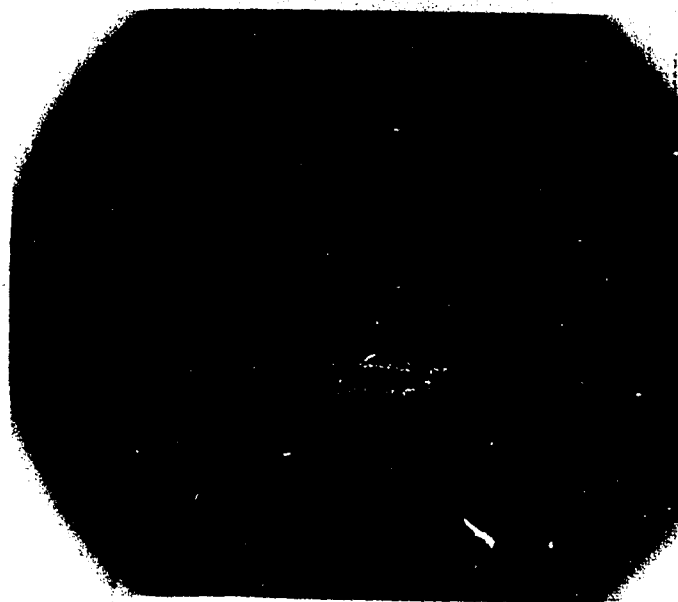


Figure 21 Phase-contrast photomicrograph of a denuded follicular rabbit oocyte incubated in the presence of re-added cumulus cells - showing first polar body - before fixing and staining 400X.

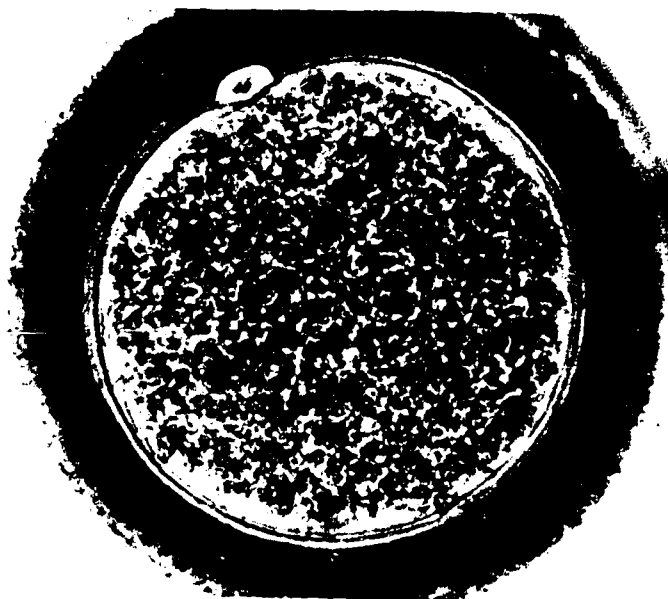


Figure 20 Phase-contrast photomicrograph of a denuded follicular rabbit oocyte incubated in the presence of progesterone - showing first polar body - before fixing and staining 400X.

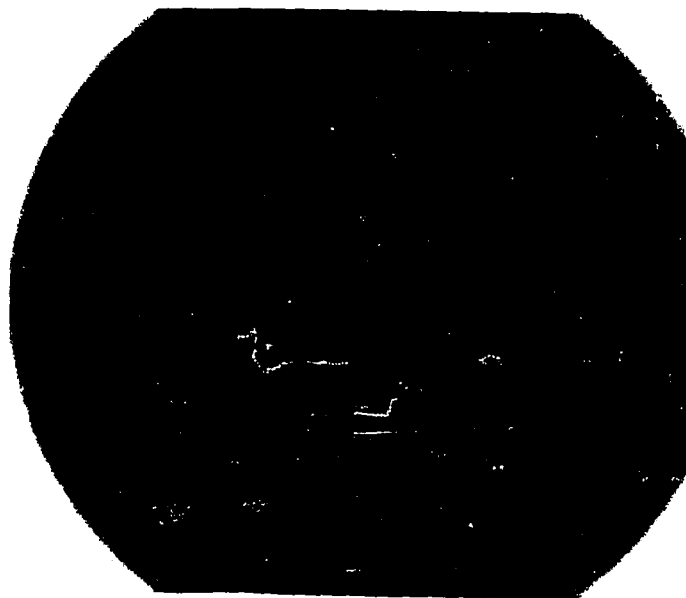


Figure 21 Phase-contrast photomicrograph of a denuded follicular rabbit oocyte incubated in the presence of re-added cumulus cells - showing first polar body - before fixing and staining 400X.

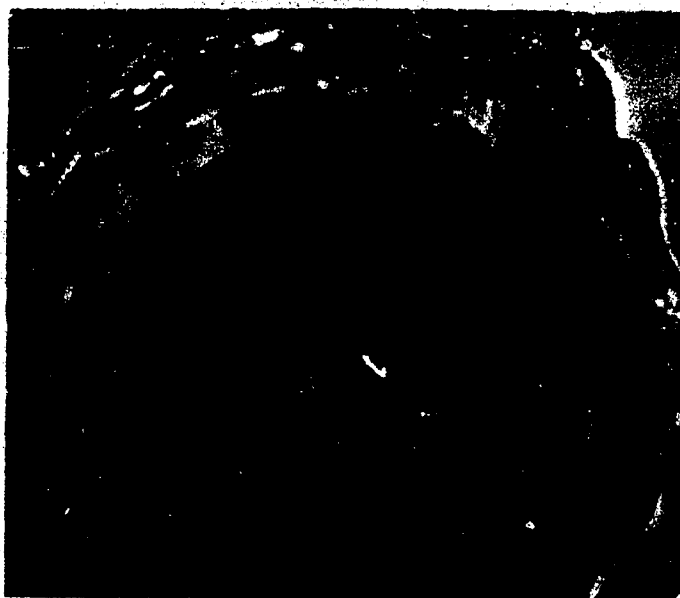


Figure 22 Phase-contrast photomicrograph of a normal follicular cow oocyte after culture for 38 hours showing first polar body - before fixing and staining 425X.



Figure 23 Phase-contrast photomicrograph of a denuded follicular cow oocyte incubated in the presence of re-added cumulus cells - showing first polar body - before fixing and staining 425X.



Figure 22 Phase-contrast photomicrograph of a normal follicular cow oocyte after culture for 38 hours showing first polar body - before fixing and staining 425X.



Figure 23 Phase-contrast photomicrograph of a denuded follicular cow oocyte incubated in the presence of re-added cumulus cells - showing first polar body - before fixing and staining 425X.



Figure 24 Phase-contrast photomicrograph of a follicular rabbit oocyte after 12 hours of culture showing cytoplasmic degeneration - unstained X425.

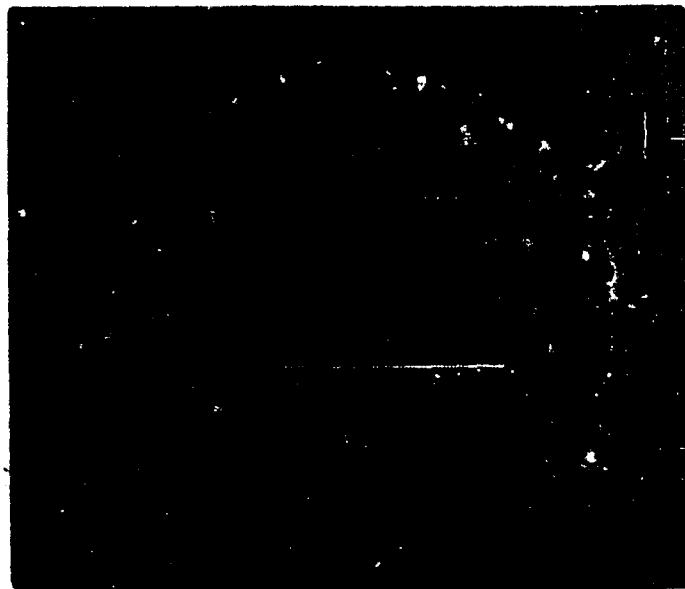


Figure 25 Phase-contrast photomicrograph of a follicular rabbit oocyte completely degenerated after 12 hours of culture - unstained X400.

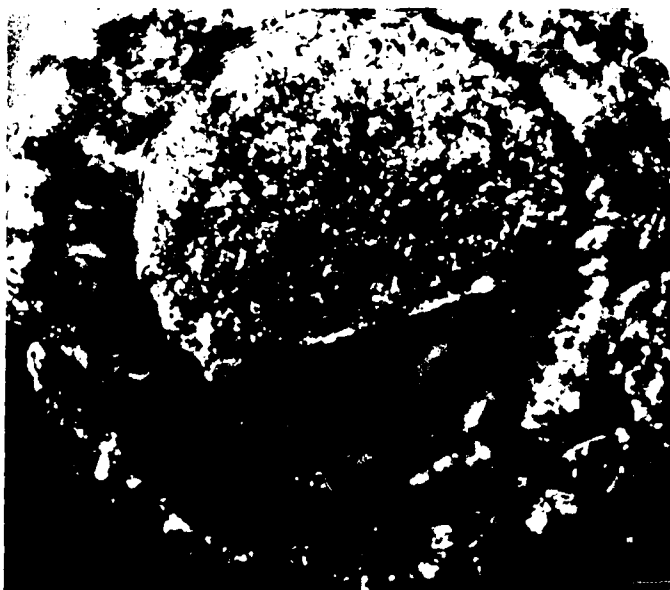


Figure 24 Phase-contrast photomicrograph of a follicular rabbit oocyte after 12 hours of culture showing cytoplasmic degeneration - unstained X425.

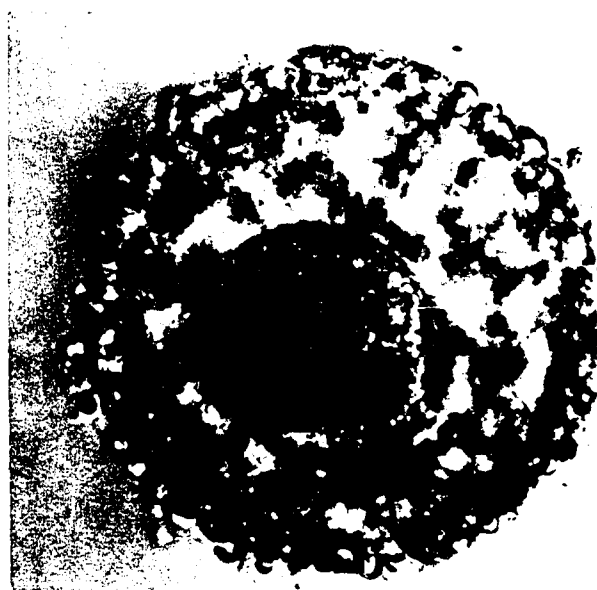


Figure 25 Phase-contrast photomicrograph of a follicular rabbit oocyte completely degenerated after 12 hours of culture - unstained X400.

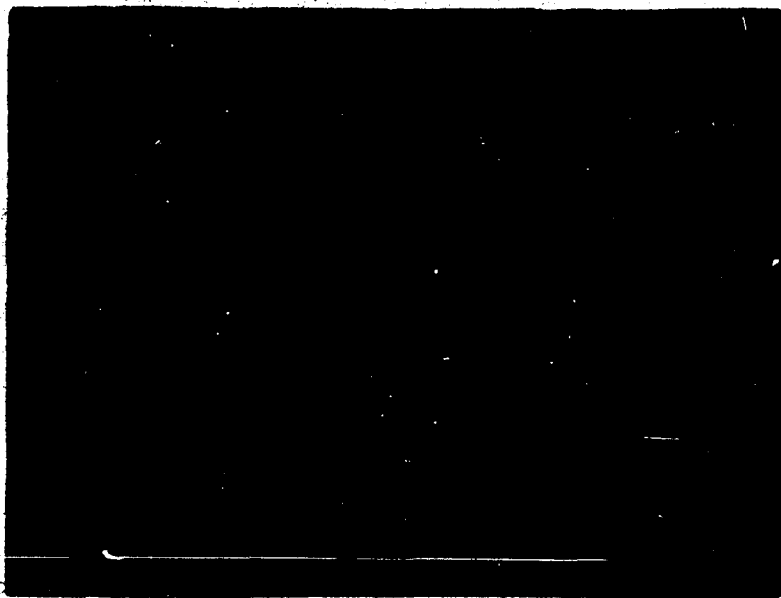


Figure 26 Phase-contrast photomicrograph of a follicular cow oocyte cultured in vitro and transferred into sheep - showing shrunken, degenerate cytoplasm - before fixing and staining 550X.



Figure 27. Vaginal speculum with lights attached during attempt to transfer oocytes into oviduct via incision in dorsal vagina.

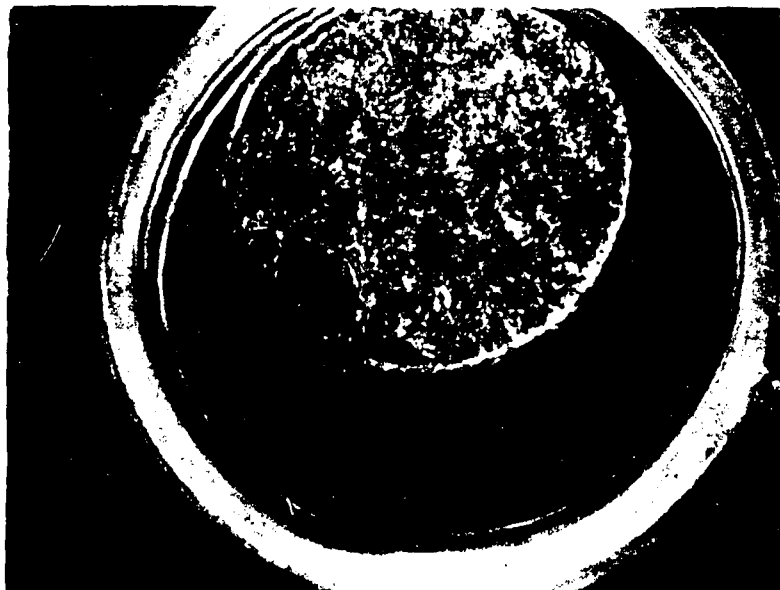


Figure 26 Phase-contrast photomicrograph of a follicular cow oocyte cultured in vitro and transferred into sheep - showing shrunken, degenerate cytoplasm - before fixing and staining 550X.



Figure 27. Vaginal speculum with lights attached during attempt to transfer oocytes into oviduct via incision in dorsal vagina.



Figure ~~Phase-contrast~~ photomicrograph of an
28 8-celled rabbit oocyte following in vitro
culture of 24 hours in 100% pig serum -
before fixing and staining 525X.



Figure ~~Phase-contrast~~ photomicrograph of a 12-
29 celled rabbit oocyte following in vitro
culture of 26 hours in 100% rabbit serum
- before fixing and staining 525X.

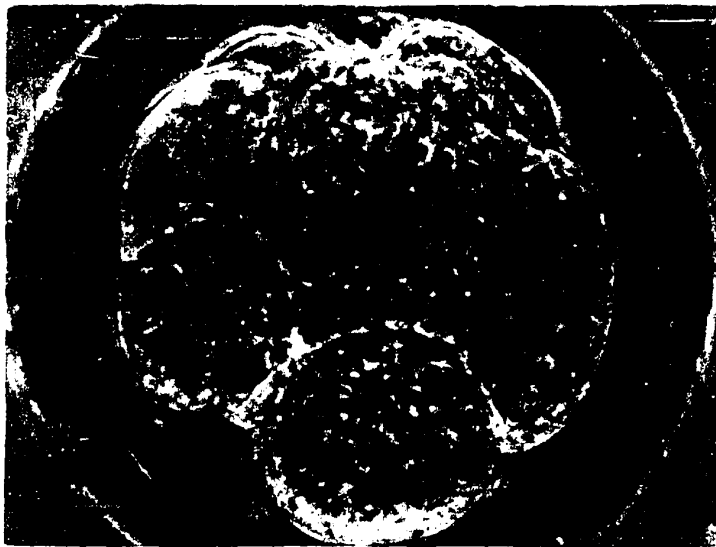


Figure 28 Phase-contrast photomicrograph of an 8-celled rabbit oocyte following in vitro culture of 24 hours in 100% pig serum - before fixing and staining 525X.

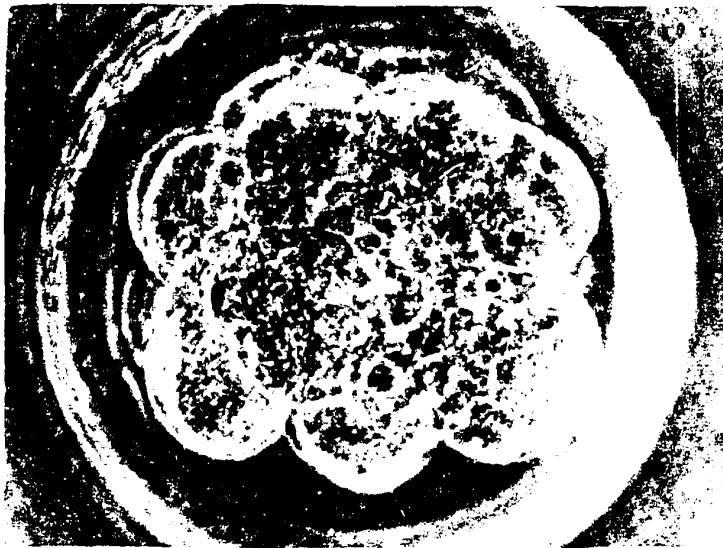


Figure 29 Phase-contrast photomicrograph of a 12-celled rabbit oocyte following in vitro culture of 26 hours in 100% rabbit serum - before fixing and staining 525X.