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The Blood-Oocyte Barrier: Morphological Evidence Implicating The Zona Pellucida

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THE BLOOD-OOCYTE BARRIER: MORPHOLOGICAL EVIDENCE IMPLICATING THE ZONA PELLUCIDA

by

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(Chairperson)

This dissertation meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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ABSTRACT

A developing oocyte is capable of synthesizing "non-self" proteins recognized as foreign by the maternal immune system. Since the oocyte may be perceived as foreign it must be protected from interaction with the immune system in order for it to mature to the point of ovulation. Oocytes that begin development but do not mature to the point of ovulation become atretic and are destroyed and phagocytized by the maternal system. This study proposes that the zona pellucida of the ovarian follicle is the barrier between the immune system and the oocyte. The suggestion is made that the oocyte may play a role in blocking the immune system during its maturation, and follicles in which the oocyte is incapable of providing for its own protection become atretic.

Indirect immunolabelling techniques employing both peroxidase and fluorochrome-conjugated antibodies were used to assess the presence of immunoglobulin G, female protein, and albumin in the ovary of the golden hamster *Mesocricetus auratus*. This experimental technique indicates that immunoglobulin G, female protein, and albumin are found localized in the zona pellucida and liquor folliculi of ovarian follicles.

Intravenous injection of fluorescein-conjugated hamster immunoglobulin G demonstrates the ability of the zona pellucida to limit access of serum immunoglobulin G to the oocyte. The labelled immunoglobulin G circulates rapidly through the animal and does not appear to be impeded in its access to the follicle. It accumulates rapidly in the

liquor folliculi and zona pellucida yet never appears within the perivitelline space.

The results of this study suggest that the zona pellucida is responsible for separating the developing oocyte from the maternal immune system, thus forming a "blood-oocyte" barrier to immunoglobulins. Further speculation is made that the oocyte plays a role in its own protection by production of factors capable of blocking complement fixation. If incapable of providing for its own protection, the oocyte is acted upon by the maternal immune system. The zona pellucida and liquor folliculi aid the immune system by providing an area for local storage of proteins necessary to accomplish complement fixation and subsequent follicular atresia.

INTRODUCTION

The gonads of all animals are complex organs which are not only subjected to hormonal regulation, but are also involved in the process of reproduction by their production of gametes and hormones.

In the male, the testis is the site of production of spermatozoa. This process is termed spermatogenesis. Spermatogenesis is a complex process which can be subdivided into four separate stages. The first and second stages involve cell division. The first stage utilizes mitosis to maintain a population of undifferentiated stem cells called spermatogonia. This step is termed spermatocytogenesis. The next stage, called meiosis, involves the ultimate production of a haploid cell, called the spermatid. These cells subsequently differentiate into spermatozoa through spermiogenesis. Meiosis involves two successive nuclear divisions with one division of chromosomes. Spermatogenesis results in the formation of 8 haploid spermatozoa from one diploid cell, the type B spermatogonia. Briefly, the process of spermatogenesis is as follows: preleptotene primary spermatocytes undergo DNA synthesis and therefore contain 4n DNA, and 2n chromosome number. These cells then enter the prophase of meiosis I. During this extended prophase there is pairing of homologous chromosomes, after which there is an interchange of chromatid segments between the homologous pairs. This interchange is termed crossing-over and ensures genetic diversity (Sadler, 1985; Dym, 1983). Meiosis I is also

termed the reductional division since the resultant cells, the secondary spermatocytes, have 2n DNA and 1n chromosome number. Secondary spermatocytes then undergo meiosis II to form spermatids. Spermatids contain 1n DNA and 1n chromosome number, and are referred to as haploid cells.

A haploid cell is no longer recognized by the body as being "self", due to the synthesis of proteins that are encoded by DNA of chromosomes formed through crossing-over. Cell surface antigens specific to adluminal spermatocytes have been identified (Millette and Bellve, 1977; O'Rand and Romrell, 1977). The antigens appear during the pachytene stage of the first meiotic prophase, and are expressed by all germ cells at subsequent stages of division. This indicates that pachytene primary spermatocytes are capable of extensive gene transcription and translation (Millette and Bellve, 1977). If spermatids produce antigenic proteins the interaction between these proteins and circulating immunocytes may induce the formation of antibodies that could lead to autoimmune phenomena. A protective mechanism must exist which limits the interaction of the proteins of the germ cell coat and the blood circulatory system. The morphological basis of such a mechanism is found at the level of the Sertoli cell and is referred to as the blood-testis barrier. As the spermatocyte undergoes meiosis I it is isolated by adjacent Sertoli cells, which send out cellular processes beneath the spermatocyte and connect to each other through tight junctions (Dym, 1973). In fact, spermatocytes are protected by the blood-testis barrier before they become true haploid cells. This subdivides the seminiferous tubule epithelium into two compartments, the adluminal

compartment which lies on the lumen side of the tight junctions, and the basal compartment which lies at the basal end of the Sertoli cell. Thus the basal compartment contains spermatogonia and preleptotene primary spermatocytes and the adluminal compartment contains the more advanced stages of germ cell differentiation (Dym, 1973). It is clear from the above that this barrier enables the Sertoli cells to maintain a microenvironment in the adluminal compartment which is favorable for germ cell differentiation.

The ovary is the female homologue of the testis and as such should be expected to have similar structures and functions. As spermatogonia are the germ cell line in the testis, oogonia are the germ cells of the ovary. Whereas the spermatogonia are constantly renewed in the testis, oogonia cease replication during fetal development. Oogonia give rise to primary oocytes which enter meiosis I during fetal life and proceed to the diplotene stage of prophase at which point their maturation is arrested, that is the cell enters a resting stage. This event occurs before, or shortly after birth and lasts until division is resumed shortly before ovulation. It should be noted that the term "resting" refers only to the meiotic inactivity, since arrested oocytes continue to synthesize RNA (Zamboni, 1971). It is during transcription and subsequent translation that unique proteins may form as a result of crossing-over and nondisjunction.

The majority of primary oocytes remain dormant in the ovary until the animal reaches puberty at which time an oocyte may resume meiosis. Meiosis I is not completed until just prior to ovulation. The resultant daughter cells are not equal in terms of cytoplasmic distribution. One daughter cell, the secondary oocyte, receives the vast majority of the cytoplasm, the other, called the first polar body, receives only a scant

amount of cytoplasm. Following ovulation the secondary oocyte proceeds to metaphase II, at which point further maturation ceases. The second meiotic division is completed only if the secondary oocyte is penetrated by a spermatozoan. (For a review of oogenesis see Moore, 1988).

It should be reiterated that, unlike the spermatozoa, at no time during its ovarian life is the oocyte considered a haploid cell. The necessity of a blood-follicle barrier (Shalgi et al., 1973) or a blood-oocyte barrier would seem to be in question based upon the above description. However, during the growth phase of oogenesis the oocytes synthesize the majority of the components that they bring to the event of fertilization. Proteins that are known to be synthesized in the growing oocyte include tubulin (Schultz et al. 1979), lactate dehydrogenase (Brinster, 1965; Mangia et al., 1976), actin and intermediate filament proteins (Kaplan et al., 1982), ribosomal proteins (LaMarca and Wassarman, 1979), and the proteins of the zona pellucida (Bleil and Wassarman, 1980a; Shimizu et al., 1983). It has been documented that the amount of protein synthesized by the oocyte when it enters its growth phase is about 38-fold that of a resting oocyte (Schultz et al., 1979). If the DNA of the oocyte has changed, due to crossing-over, then proteins may be produced that will be recognized as foreign by the host (maternal) system. As with the testis, interaction of these proteins with the maternal blood may result in the production of antibodies that could lead to autoimmune phenomena.

The zona pellucida (ZP) is an amorphous glycoprotein coat which is formed during the development of the oocyte, surrounds the oocyte and later the blastocyst, and remains intact until implantation (Dunbar, 1983a). It first appears at the primary unilaminar follicle stage and is considered to

be fully formed by the primary multilaminar follicle stage. Because the molecules of the ZP are produced and secreted during the early stages of oocyte growth and development, they provide excellent markers for this dynamic process (Oakberg and Tyrell, 1975; Wolgemuth et al., 1984; Philpott et al., 1987).

The zona pellucida is first seen at the light microscopic level at the time when the cells surrounding the primary oocyte change from a rather flat, squamous cell type to a single layer of cuboidal cells (Dietl, 1989). The ZP does not form as a complete and continuous extracellular membrane around the entire circumference of the oocyte but rather as discontinuous portions which fuse only secondarily to constitute a complete encasement (Dietl, 1989). The ZP is first recognized as amorphous material lying between the pocket-shaped protrusions of microvilli of the oocyte membrane and follicular cells. Initially it is found only very sparsely distributed in the perivitelline space. The primary appearance of ZP material coincides with an increased number of microvilli of the oocyte and cell protrusions of granulosa cells. The microvilli extend into the substance of the ZP and tend to be more numerous than the protrusions of the adjacent follicle cells. Whereas the plasma membrane of the resting oocyte shows only a few short microvilli, they become progressively more numerous and uniformly distributed over the surface of the oocyte concomitant with the formation of the ZP. Morphologically the maturation of ZP begins when amorphous granules appear between the oocyte and hypertrophied follicular cells. Greve and Wassarman (1985) identified these glycoprotein granules as zona protein one (ZP1) which has cross-linking properties with individual filaments within the ZP of the mouse.

The ZP consists of randomly arranged, fibrillogranular materials that give rise to its amorphous appearance observed in scanning electron micrographs (Phillips and Shalgi, 1980) and in transmission electron micrographs (Wassarman and Josefowicz, 1978; Dietl and Czuppon, 1984). There are fine fibrillogranular connections within the developing ZP matrix which seem to serve as filament branch points. Perhaps these fine granular particles are involved in the branching of ZP filaments. A greater degree of polymerization occurs with ZP growth and the formation of a glycoprotein network takes place. Ultimately the density of the mature ZP fibrils increases. There is, however, a difference in density in the mature ZP between the inside (facing the oocyte) and the outside (facing the follicular cells): the fibrils being more densely packed inside than outside (Dietl and Czuppon, 1984). Further studies have demonstrated that the ZP of some species appear to contain multiple layers (Hope, 1965; Wolgemuth et al., 1984).

The origin of the ZP has been the subject of some debate, with some authors implicating the granulosa cells as the source (Guraya, 1974) while others choose the oocyte as the source (Tesoriero, 1977; Haddad and Nagai, 1977; and Bleil and Wassarman, 1980b). Some consider the ZP to be the product of both the granulosa cells and the oocyte (Chiquoine, 1960; Hertig and Adams, 1967).

Indirect evidence exists that granulosa cells produce the ZP matrix. When rabbits are immunized with porcine ZP proteins, the oocytes are destroyed at the stage at which they begin to initiate formation of the ZP matrix (Skinner et al. 1984). Even though there are no remnants of oocytes, there are distinct matrices which stain identical to ZP surrounded

by "granulosa" cells. These matrices are easily distinguished from the collapsed remnants of ZP from atretic follicles which do not contain granulosa cells within the center of the collapsed ZP. It therefore appears from these studies that at least some ZP proteins are produced in the absence of the oocyte in vivo (Dunbar et al., 1989). Additional evidence for the synthesis of ZP glycoproteins by granulosa cells is provided by immunocytochemical localization of ZP glycoproteins in the follicle of the rabbit (Wolgemuth et al., 1984).

On the other hand, the oocyte appears to be the primary producer of the ZP. Bousquet et al. (1981) found that a ZP antigen originating from the oocyte begins to be produced at the stage of the primary follicle. Studies of the molecular details of the genes that code for the mouse ZP proteins show that these proteins are synthesized only by the oocyte genome (Chamberlin et al., 1989). Further studies (Dietl, 1989) indicate that in mouse zona protein three (ZP3) expression appears to be oocyte specific and limited to the growth phase of oogenesis. Fully grown oocytes do not appear to make ZP proteins (Bleil and Wasserman, 1980b), and oocytes from superovulated animals, although still synthesizing proteins, no longer synthesize ZP proteins (Shimizu et al., 1983).

Using an immunohistochemical method, Leveille et al. (1987) indicated that the native anti-ZP antibody binding material of hamster ZP is synthesized in the oocyte itself and not in the granulosa cells. Immunocytochemical localization methods have been used to identify ZP antigens in the cytoplasm of hamster oocytes (Leveille et al. 1987) and rabbit oocytes (Wolgemuth et al. 1984). These studies indicate that at least a portion of the ZP is produced in the oocyte.

Many investigations attempted to characterize the constituent macromolecules of the ZP. These studies led to the conclusion that there are marked variations in the morphological, biochemical, immunochemical, and physiochemical properties of the constituent glycoproteins of the ZP in different mammalian species (Bleil and Wassarman, 1980 a-c; Dunbar et al., 1981, 1985; Sacco et al. 1981; Drell and Dunbar, 1984; Timmons et al., 1987; Yurewicz et al., 1987; Maresh and Dunbar, 1987).

A major morphological variation occurs in the thickness of the ZP between different mammalian species (Dunbar, 1983a). The ZP surrounding fully grown mouse oocytes is 7μm thick and contains 3-4 ng of protein, made up of three major glycoproteins termed zona protein one (ZP1), zona protein two (ZP2) and zona protein three (ZP3) (Bleil and Wasserman, 1980a; Shimizu et al., 1983). The three proteins appear to be organized in a matrix of branched filaments (Greve and Wasserman, 1985). Certain chemical and enzymatic treatments, specifically dithiothreitol and chymotrypsin, reduce the branched matrix to individual filaments composed of both ZP2 and ZP3, suggesting that ZP1 is responsible for the branching (Greve and Wasserman, 1985). Thus, the ZP of a fully grown oocyte appears to be an ordered structure of filaments composed of ZP2 and ZP3, either together or separate, and held together by ZP1.

Hamster ZP proteins in reduced state are resolved into two components of apparent molecular weights of 80 kd and 240 kd by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). By a second gel

electrophoresis in the same direction without SDS, a third band of an apparent molecular weight of 150 kd appears (Ahuja and Bolwell, 1983).

Three major families of ZP proteins from human ZP (ZP1, 80-120 kd: ZP2, 73 kd: ZP3, 59-65 kd) and rabbit (ZP1, 100-118 kd; ZP2, 83-110 kd; ZP3, 80-92 kd), and four major families from squirrel monkey (ZP1, 63-78 kd; ZP2, 63-70 kd; ZP3, 47-51 kd; ZP4, 43-47 kd) can be separated by two-dimensional SDS-PAGE under reducing conditions. A similar result was obtained with rabbit ZP proteins by Dunbar et al. (1981). As previously described, ZP1 is responsible for cross-linking the fibrillar ZP2 and ZP3. Recently, Wassarman (1987a,b) described receptors present in ZP3 which are responsible for species specificity of sperm binding. Recently a fourth glycoprotein has been isolated from the ZP of oviductal eggs from the golden hamster. This glycoprotein, termed zona protein zero (ZP0) is produced by the epithelial cells of the oviduct and was found to be in the weight range of 200-240 kd (Oikawa, 1989; Sakai et al., 1988). During in vitro fertilization studies, it was found that antibodies raised to ZP0 greatly inhibited sperm binding (Sakai et al., 1988).

The permeability of the ZP was the subject of ongoing study for many years. Austin and Lovelock (1958) believed that the ZP of the rabbit, rat, and hamster are permeable to substances with a molecular weight of 1,200 or less. This meant that the vitellus could be considered directly accessible to all the known essential food components including vitamins, to the majority of pharmacologically active compounds, and to all natural steroid hormones. It would not be accessible to most enzymes, antigens, antibodies, or protein hormones (Austin, 1961). It was later

demonstrated (Anderson, 1967) that horseradish peroxidase (mol. wt. 40,000) penetrated through the ZP in the ovarian oocyte of the rabbit.

Simmons and Russell (1966) suggested that the ZP may be impermeable to immunoglobulins, thereby protecting the embryo from the potentially adverse effects of the maternal immune system. Heyner et al. (1969) provided support for this theory, stating that immunoglobulins (mol. wt. 150,000 - 900,000) might not be expected to pass through the ZP. This statement is based on the observation that the development of eight-cell mouse eggs to the blastocyst stage is uneffected by the presence of cytotoxic antibody plus complement *in vitro*. However, Sellens and Jenkinson (1975) indicated that the ZP of the mouse blastocyst was permeable to high molecular weight immunoglobulins based on their observation that ZP-enclosed embryos were destroyed when exposed to serum fractions containing either IgG or IgM and complement.

The inner layers of the follicular cells, the granulosa cells, maintain specialized contacts with the oocyte and assist in protein and nucleic acid synthesis via metabolic transfer to the oocyte (Anderson and Albertini, 1976; Gilula et al., 1978; Heller et al., 1981; Brower and Schultz, 1982). This may give a false impression as to the permeability of the ZP.

While a considerable amount of information is now available on the structure and function of the mature ZP obtained from large ovarian follicles or from ovulated ova with respect to their potential roles in the fertilization process, few studies have been carried out to evaluate the potential roles of the ZP proteins during follicular development or during the onset of sexual maturation (Dunbar et. al., 1989).

The ZP-encased oocyte residing in the developing follicle is bathed in follicular fluid, a substance very similar in composition to serum and previously demonstrated to contain immunoglobulins (Matousek et al., 1986; Kay et al., 1985; Hussein and Bourne, 1984). The protein content of human follicular fluid compares to that of blood serum (Manarang-Pangan and Menge, 1971; Shalgi et al., 1973; Perricone et al., 1990). Although their results differed in respective ratios, all agreed, along with Clark (1900) that follicular fluid appears to be mainly derived from blood by transudation and not from granulosa cells. This provides evidence that the basement membrane of the follicle presents little if any barrier to the diffusion of substances from the theca into the follicle.

Perricone et al. (1990) found that the human follicular fluid contained functionally active complement proteins in amounts similar to those present in normal human serum. Numerous investigations (Sacco, 1981; Dunbar, 1983b) involving immunization of females with ZP antisera indicated that ZP antibodies can indeed reach and combine with the ZP in the follicle. ZP-encased eggs obtained from such immunized females have all possessed ZP antibody on the ZP surface, as established by microscopic or immunofluorescent procedures (Sacco, 1979; Sacco et al., 1983). This indicates that the ZP is accessible to blood-borne proteins.

Shalgi et al. (1973), using Ouchterlony double diffusion tests, reported the presence of both IgG and IgA in human follicular fluid. Proteins with a molecular weight of 150,000, such as IgG were present in lower concentrations than normal serum. Proteins of higher molecular weight, such as IgM (900,000 mol. wt.) were undetectable in follicular fluid, or detectable in trace amounts only. The oocyte is not in direct

contact with the follicular fluid, but rather is separated from it by the ZP and the cumulus oophorus. Since follicular fluid was found to contain components of the complement fixation pathway, the ZP may be the only remaining barrier for protection of the oocyte.

The ability of molecules to pass through the ZP may depend not only on molecular weight, but also on molecular configuration, degree of aggregation, or the charge characteristics of the molecule in relation to the ZP itself. Hastings et al. (1972) stated that the ZP might exclude some molecules on the basis of binding with these molecules or possibly by charge effects. This was supported by experiments which suggested that in the rat and mouse the ZP contained compounds that could give rise to a net negative charge within the ZP which would impede the entry of molecules also bearing a net negative charge (Sellens and Jenkinson, 1975).

Few studies have been conducted on the antigenic composition of mammalian ovaries and eggs, however, with the advent of in vitro fertilization many investigators have focused their research on this area. Autoantibodies to the ZP have been found in the serum of infertile women (Shivers and Dunbar, 1977; Mori et al., 1979; and Buckshee and Mhaskar, 1984). These findings provide further evidence that the ZP is accessible to the serum proteins of the maternal blood circulatory system. This is an indication that the ovary and its components can be affected by the maternal immune system.

If the interaction between the immune system and the ovary, and more specifically the follicle and oocyte, are to be investigated, it becomes necessary to provide some background information on aspects of the immune system deemed pertinent to this study.

Immunoglobulins are glycoproteins composed of 82-96% polypeptide and 4-18% carbohydrate. The polypeptide component possesses almost all of the biological properties associated with antibody molecules. Antibodies (immunoglobulins) are bifunctional molecules in that they bind specifically with antigens and also initiate a variety of secondary phenomena, such as complement fixation and histamine release by mast cells, which are independent of their specificity for antigens.

Each immunglobulin contains at least one basic unit, or monomer, comprised of four polypeptide chains. One pair of identical polypeptide chains contains approximately twice the number of amino acids, i.e. is approximately twice the molecular weight, of the other pair of identical polypeptide chains. The chains of higher molecular weight are thus designated heavy (H) chains and those of lower molecular weight designated light (L) chains. Each polypeptide chain contains an amino-terminal portion and a carboxy-terminal portion, the former termed the variable (V) region and the latter termed the constant (C) region. These terms denote the heterogeneity or variability in the amino acid residues between the two regions.

The part of the antibody molecule that binds antigen is formed only by a small number of amino acids in the V region of H and L chains and these amino acids are brought into close relationship by the folding of the V region.

There are five classes of immunglobulins, designated IgG, IgA, IgM, IgD, and IgE. They are defined by antigenic differences in the constant regions of the heavy chains.

Enzymatic digestion of an IgG molecule using papain yields two antigen-binding fragments (Fab) and one crystallizable fragment (Fc). The digestion by papain takes place in an area of the molecule known as the hinge region. The hinge region is defined as the area of the H chain in the C region between the first and second C region domains. This area is more flexible and is more exposed to enzymes and chemicals.

Enzymatic digestion of a molecule of IgG using the enzyme pepsin yields one F(ab)'2 molecule and small peptides. The F(ab)'2 molecule is composed of two Fab units and the hinge region, with intact inter-H chain disulfide bonds. The later are left intact since pepsin cleaves the molecule on the carboxy-terminal side of these bonds (for a review of immunoglobulins see Goodman, 1987).

In adult humans IgG constitutes approximately 75% of the total serum immunoglobulin. The IgG class is made up of four subclasses designated IgG1, IgG2, IgG3, and IgG4. Immunoglobulin G is the only class of immunoglobulin that is known to cross the placental barrier and this phenomena is believed to be a receptor mediated event (Goodman, 1987; Steward, 1974). Immunoglobulin G Fc receptors have been isolated and it is believed that these receptors play a role in the transport of IgG (Van de Winkel and Anderson, 1991). Rodewald and Kraehenbuhl (1984) described a receptor-mediated endocytosis in which large amounts of intact IgG were transported across the intestinal epithelium.

It is interesting to note that IgG does not appreciably cross the choriocapillaris of the choroid to accumulate in Bruch's membrane (Pino and Thouron, 1983). This suggests that capillaries in general may not necessarily be permeable to IgG. There is a several-fold rise in the

microvascular permeability to IgG in the testis of rats at 27-30 days of age (Pollanen and Setchell, 1989). It is interesting that this rise in permeability coincides with the beginning synthesis of an interleukin-1-alpha-like factor by the Sertoli cells (Syed et al., 1988). Interleukin-1 is known to regulate the functions of endothelial cells in other tissues (Bevilacqua et al., 1985).

Two additional proteins that are pertinent to this study are albumin and female protein (FP). Serum albumin is a highly flexible molecule containing 575 amino-acids combined to give an overall molecular weight of 65,000. Albumin is characterized by its extreme solubility in water, its negative charge of 19 at pH 7.4, and its lack of a carbohydrate moiety (Rothschild et al., 1972). It is known that in addition to its role in maintaining the colloid osmotic pressure of plasma and interstitial fluid, albumin is an important carrier for various molecules such as fatty acids, testosterone, estradiol, and thyroid hormones. These molecular complexes (ligands) are then presented to the surfaces of the target cells (Corvol and Bardin, 1973; Hutter et al., 1984; Ockner et al., 1983).

Estimates of the lymph/plasma albumin ratio revealed that the amount of albumin passing from the plasma into interstitial fluid varies significantly from organ to organ (Everett and Simmons, 1958; Pino and Thouron, 1983; Sharpe, 1979; Williams and Greener, 1984; Williams et al., 1981; Yokota, 1983). Considering the different types of endothelium found in these various organs, no firm correlation can be established between the endothelial structure and the rate of albumin transport. Other properties of the endothelial cell may account for the difference in the rate, speed, and kinetics of albumin uptake between various tissue (Ghitescu et al., 1986). Albumin transport through endothelial cells via transendothelial

channels (connective transcytosis) and plasmalemmal vesicles (vectoral transcytosis) has been described (Ghitescu, 1986; Simionescu, 1981). Sage et al. (1984) described a unique albumin binding glycoprotein synthesized by bovine, porcine, and human endothelial cells in culture.

Albumin binding sites have been demonstrated on the surfaces of liver and heart cells (Ockner et al., 1983; Hutter et al., 1984) as well as for endothelial cells of the capillaries (Ghitescu et al., 1986; Schnitzer et al., 1988). The binding of the albumin-ligand complex to membrane receptors of tissues triggers endocytosis of the hormone-receptor complex either with or without the internalization of the albumin (Szego, 1975; Shteri et al., 1982; Ockner et al., 1983). Albumin may also be significant in its ability to remove ligands from target cells, and by this mechanism may mediate functions of steroid-producing and steroid-responding cells (Krishna and Spanel-Borowski, 1989).

Female protein (FP) is an alpha globulin found in the serum of the female syrian (golden) hamster, *Mesocricetus auratus* (Coe, 1977; Coe et al., 1981). Female protein is also found in the Kurdistan hamster, *Mesocricetus brandti*, but is not found in Chinese, Armenian, European, or Djzungerian hamsters (Coe et al., 1981). Female protein, produced in the liver (Coe and Ross, 1985), is a pentamer approximately 13 nm in diameter and has a molecular weight of 150,000. The native molecule consists of five 30,000 mol. wt. subunits noncovalently assembled to form the 150,000 mol. wt. parent molecule (Coe et al., 1981).

Although FP is detected in conspicuous amounts in normal female hamster serum (2mg/ml), it is not detectable by simple gel diffusion in normal serum from adult males (Coe et al., 1981). The protein is,

however, detectable for a transient period in young male hamsters (Coe, 1977; Coe et al., 1981).

Castration of adult males results in a 50-fold increase in the serum levels of FP (Coe and Ross, 1990), while administration of testosterone to castrated males results in a concomitant decrease in serum levels of FP (Coe, 1977; Coe et al., 1981; Coe and Ross, 1990). This would indicate a suppressor function of the testicles via testosterone on serum FP in adult male hamsters.

Ovarectomized (castrated) female hamsters show no significant drop in serum levels of FP, indicating that intact ovaries are not required for maintenance of normal serum FP levels. Concomitantly, female hamsters injected with testosterone show a significant decrease in serum FP levels, indicating that testosterone has a suppressor effect on serum FP in females as well as males, i.e. a primary suppressor effect of an androgen (Coe, 1977; Coe et al., 1981).

Female protein is remarkably similar to two other serum proteins found in other animals, C-reactive protein (CRP) and serum amyloid protein component (SAP). In fact, FP appears to represent the CRP-SAP homologue in syrian hamsters (Coe et al., 1981). In contrast to FP, CRP and SAP are normally present in low serum concentrations and are not known to be under sex-hormone control (Coe, 1977). Amino-terminal sequence data shows a clear homology between FP and SAP in which 19 of 23 identifiable residues (83%) were identical. Since CRP and SAP have been shown to be homologous (Osmand et al., 1977; Levo et. al., 1977; Coe and Ross, 1983) and FP and SAP exhibit 83% homology, the three

proteins appear to have evolved from the same ancestral gene (Coe et al., 1981).

Human CRP is the classic acute-phase reactant, which can increase almost 1000-fold in serum levels after nonspecific inflammatory stimuli (Coe et al., 1981). Numerous biological functions have been shown for CRP including activation of complement (Siegel et al., 1974; Kaplan and Valanakis, 1974), interaction with T lymphocytes (Croft et al., 1976) and platelets (Fiedel and Gewurz, 1976), and enhancement of phagocytosis (Kindmark, 1971; Pepys et al., 1978). In contrast, SAP is found in normal human and mouse serum, but only in mice does it increase as a typical acute-phase reactant after inflammatory stimuli (Pepys et al., 1979).

Once bound to C3, CRP activates the classical complement pathway via C3 conversion independent of the presence of specific antibody or immunoglobulin (Kaplan and Volanakis, 1974; Pepys et al., 1978). The CRP analog in the rabbit, CxRP, was shown to stimulate leukocyte migration (Wood, 1951), facilitate phagocytosis (Hokama et al., 1962; Kindmark, 1971, 1972), signal enhanced immune response (Wood, 1953), and induce lymphocyte-blast formation (Hornung and Friechi, 1971).

Hamster FP, the homologue of CRP and SAP, exhibits some acute-phase reactivity. In male hamsters there is a moderate increase in serum FP after nonspecific inflammatory stimuli (Coe, 1977; Coe et al., 1981). However in female hamsters FP serum levels show a dramatic decrease in serum levels during an acute-phase response (Coe and Ross, 1983). Hamster FP also activates the classical complement pathway at the level of C1 (Coe and Ross, 1985). Immunoglobulin G and female protein are both implicated as initiators of the complement fixation pathway.

Complement is a complex biological system made up of nine protein components, designated C1-C9. The first of these components, when activated by immune complexes, activates the next component which in turn activates the next component in the sequence and so on thus producing a cascade effect. The end result of complement fixation is lysis of cell membranes leading ultimately to cell death. In addition to its role in cell lysis, the complement system also produces proteins which promote chemotaxis, i.e. the attraction of polymorphonuclear phagocytes, as well as proteins which enhance opsonization.

Briefly, the cascade system is described by Steward (1974). The initial step in the activation of the complement sequence by antigen-antibody complexes is the binding of C1 to active sites on the Fc region of the antibody which has been produced as a result of immune complex formation. The C1 recognition unit consists of three subunit types: C1q, C1r and C1s, and the reaction with the altered antibody occurs through the C1q component, which can bind up to five IgG molecules. This binding results in C1r activating C1s. The active form of C1s is an enzyme the substrates of which are components C4 and C2. It first catalyzes the binding of C4 to the cell and then the binding of C2 to the bound C4. This complex (C42) is termed C3 convertase which has C3 as its substrate. At this stage two events occur as a result of C3 convertase activity on C3. Fragments of C3 called C3a are released and these have anaphylotoxin activity, for histamine release, and also are chemotactic for polymorphonuclear phagocytes. The major fragment of C3, called C3b, is transferred to the target cell membrane, and is recognized by the C3 binding sites of macrophages leading to immune adherence and subsequent

phagocytosis. C3b also modifies the C42 enzyme to produce the C423 enzyme. This acts on C5 which then interacts with C6 and C7 resulting in the binding of C567 to the membrane. Also at this stage, further fragments with chemotactic and anaphylatoxin activity are produced. As previously described, it is known that the complement fixation pathway need not be activated by an antigen-antibody complex, but rather may be activated by proteins such as CRP, CxRP, SAP, and FP. Finally C8 and C9 become attached to the membrane and a lesion is produced. (For an in depth review of the complement fixation pathway see Cooper, 1987).

Since the oocyte develops in a manner similar to the spermatocyte, and the spermatocyte has been shown to produce surface proteins that are antigenic to the maternal immune system, one might assume that the oocyte also produces proteins specific to its cell coat. These proteins would label the oocyte as a foreign cell, thus a barrier between these cells and the maternal immune system is essential. This study proposes that the ZP provides the morphological basis of this blood-oocyte barrier. Further speculation is made that the oocyte plays a role in its own protection by production of complement inhibitors.

MATERIALS AND METHODS

Female golden hamsters (*Mesocricetus auratus*) were kept on a 14:10 light dark cycle at 22°C. Only hamsters that had three consecutive 4 day estrous cycles were used in these studies. Ovulation occurs late on day four of the estrous cycle. Day one of the estrous cycle is characterized by a conspicuous vaginal discharge. Animals were checked for discharge between 0700 and 0900 hours. All animals used in this study were in the weight range of 108-230 grams. Animals were anesthetized by intraperitoneal (i.p.) injection of phenobarbitol (Nembutal) at 10mg/100g body weight.

Indirect Immunolabelling Technique

Hamster ovaries were harvested every four hours throughout the estrous cycle beginning at 0200 hours on day one. At the appropriate time, animals were injected i.p. with Nembutal (10mg/100g body weight). Upon deep anesthesia, the thoracic and abdominal cavities were opened by a single incision. An 18 gauge pediatric i.v. cannula was passed down the descending aorta to the level of the diaphragm. The right atrium was incised to allow for drainage of blood and perfusate from the animal.

Infusion was carried out via a Harvard infusion pump equipped with a 50ml disposable syringe. Perfusion via an infusion apparatus was chosen

over injection by hand because the former allows for control of temperature and intraarterial pressure, is documentable, and is reproducible whereas manual injection does not provide this regulation (Christofferson and Nilsson, 1988). Approximately 50ml of 0.1M phosphate (PO_4) buffer (pH 7.4) was perfused at the rate of 15.3 ml/minute to allow for flushing of the blood from the vessels. This was immediately followed by approximately 150ml of 1% paraformaldehyde in 0.1M PO_4 buffer (pH 7.4) to obtain optimal preservation of antigenic tissue sites. Ovaries were immediately removed, sectioned into four pieces, and further processed using a modification of the technique used by Beckstead (1985). This technique employed the use of a glycol methacrylate embedding medium, which provides for better morphology than can be obtained using paraffin. The technique, carried out at 4°C, is as follows: 1) fix for a minimum of 6 hours in 4% paraformaldehyde in 0.1M PO_4; 2) wash for one hour in 0.1M PO_4 buffer with 2% sucrose and 50mM NH_4Cl, repeat twice; 3) wash for one hour in 0.1M PO_4 buffer with 50mM NH_4Cl, repeat once; 4) place in JB4 Plus (glycol methacrylate) infiltration medium for a minimum of eight hours; 5) embed with JB4 Plus embedding medium (Polysciences, Inc.) in number 0 gelatin capsules. A ratio of 1:10 catalyst (organic peroxide) to infiltration medium was found to yield blocks of a desirable hardness for sectioning. Upon complete polymerization the tissue was sectioned at approximately 3μm on a Sorval Porter-Blum MT2-B ultramicrotome using glass knives. The sections were placed on a drop of water on glass microscope slides which were then allowed to air dry overnight.

Immunohistochemical procedures were performed in the following order and, unless otherwise specified all incubations were carried out in a dark, moist chamber at room temperature. Each successive step was followed with a rinse of phosphate buffered saline (PBS): 1) etch plastic with 10% H_2O_2 for 15 minutes to expose tissue; 2) apply 0.25% porcine trypsin in Saline A (Gibco, Santa Clara, CA), pH 7.6 for 10 min at 37°C to unmask cross-linked binding sites (Huang, 1976); 3) apply carrier solution for 30 minutes to bind any nonspecific binding sites; 4) apply primary antibody diluted at 1:400 in carrier solution. Primary antibodies used were goat anti-hamster IgG or goat anti-mouse IgA (Cappel); 5) apply carrier solution for 30 minutes; 6) apply secondary antibody for 1 hour. Secondary antibodies used were rabbit anti-goat IgG peroxidase-conjugated Fab2 fragments diluted at 1:20 in carrier solution, or rabbit anti-goat IgG conjugated with fluorescein isothyocyanate (FITC) diluted at 1:32 in carrier solution; 7) coverslip FITC treated slides with mounting medium and seal the edges with nail polish.

Peroxidase conjugated tissue was rinsed with PBS and the peroxidase reaction was developed as follows: 1) incubate with 0.01% (0.5mg/ml) 3,3'-diaminobenzidine (DAB) in 0.1M phosphate buffer for 10 min at 37°C; 2) incubate with 0.01% DAB in 0.1M phosphate buffer with 0.1% H_2O_2 for 5 min at 37°C; 3) counterstain with Harris' hematoxylin for 6 min, blue in tap water for 2 min, dehydrate in ascending grades of ethanol and coverslip using Permount.

The carrier solution was prepared by mixing 1ml of normal serum (serum from the same species in which the secondary antibody was produced), one gram of bovine serum albumin, 1.5ml of 20% Triton

X-100, and 97.5ml of PBS according to A Practical Guide to Immunohistochemistry, published by Pel-Freez Clinical Systems. The bovine serum albumin aids in binding to nonspecific binding sites and the Triton X-100 is used to disrupt cellular membranes.

Mounting medium used for coverslipping fluorescent labelled tissue was a mixture of 2.42g tris-base/100 ml distilled water at pH 8.5, 12ml Tris at pH 8.5, 4.76ml dimethylsulfoxide (DMSO), 6ml methanol, and 2.4g of 20.30 polyvinyl alcohol (PVA). Just before use, p-phenylenediamine (PPD) was added at 1mg/ml to prevent photobleaching (Soo Siang Lim-Spiker, personal communication). After coverslipping, the edges were sealed with nail polish. A minimum of two hours should pass before viewing. It is also important to store slides prepared for fluorescence microscopy in a cool, dark container, to prevent premature decay of fluorescence.

Controls for the immunohistochemical staining procedure were conducted using an antibody substitution technique in which PBS was substituted for antibody. Three controls were carried out such that one slide was incubated with primary antibody only, one was incubated with secondary antibody only, and one was incubated having no exposure to antibody.

A series of immunolabelling experiments was also carried out on selected tissue using rabbit anti-hamster IgG, rabbit anti-hamster female protein, and rabbit anti-hamster albumin as the primary antibody diluted 1:400 in carrier solution. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG diluted 1:40 in PBS or peroxidase-

conjugated goat anti-rabbit IgG diluted 1:20 in carrier solution was employed as the secondary antibody.

An alternate technique for immunohistochemical labelling employing microwave irradiation was adapted for use on tissues. This technique has been used in the past with some success and greatly reduces the time required for immunohistochemical staining (Chiu and Chan, 1987, Leong and Milios, 1986, 1990). Microwave irradiation was carried out in a Litton Minutemaster, 700 watt microwave oven equipped with a Nordic Ware Micro-Go-Round Plus carousel, with roasting tray and cover. Slides were placed on a Plexiglass sheet in the roasting tray and distilled water was poured into the tray to completely cover the bottom. This allowed for even heating of the tissue while providing a moist, covered environment.

Immunohistochemical procedures were performed in the following order, with each successive step followed by a PBS rinse: 1) apply 10% H_2O_2 and microwave on setting one for 1 minute 10 seconds, let stand for 3 minutes 50 seconds; 2) apply 0.25% porcine trypsin in saline A and microwave on setting one for 1 minute 10 seconds, let stand for 3 minutes 50 seconds; 3) apply carrier solution and microwave on setting three for 1 minute 10 seconds; 4) apply primary antibody diluted to 1:400 in carrier solution, microwave on setting two for 1 minute 10 seconds; 5) apply secondary antibody diluted to 1:20 in carrier solution, microwave on setting two for 1 minute 10 seconds, let stand for 3 minutes 50 seconds.

Fluorescent labelled tissues were then coverslipped with mounting media as previously described, and peroxidase treated slides were developed at 37°C in a water bath, counterstained, and coverslipped with permount as previously described.

The microwave immunohistochemical staining procedure provided results similar to the original room temperature technique requiring one third of the time, however the labelling did not appear to be consistent from one section to another nor was the tissue evenly labelled.

The peroxidase treated tissue was examined and photographed on a Olympus BH-TU microscope equipped with an 80a (daylight) filter. FITC and TRITC treated tissues were examined for fluorescence and photographed on a Nikon Microphot FX Episcopic Fluorescent Microscope equipped with a Nikon B-1E filter cube for viewing FITC and a Nikon G-1B filter cube for viewing TRITC. The B-1E filter has the following illumination properties: excitation filter = 470-490 nm, barrier filter = 520-560 nm, dichroic mirror = 510 nm. The G-1B filter has the following properties: excitation filter = 546 nm, barrier filter = 590 nm, dichroic mirror = 580 nm.

To rule out the possibility of this phenomenon being a staining artifact of the ovary, ovulated oocytes were stained using the same peroxidase techniques previously described. Oviductal oocytes and flushed oocytes were studied and in each case these oocytes were found to have what appeared to be an intact ZP. Oviductal oocytes were localized by sectioning through the oviduct harvested from an animal on day one of the estrous cycle. The oviduct had been fixed and embedded in a manner identical to the ovaries, as previously described.

Ovulated oocytes were obtained by flushing the oviducts of a superovulated hamster. Superovulation was accomplished according to the method of Kan et al. (1990). Briefly, 25 international units (IU) of pregnant mare serum gonadotropin were injected i.p. at 0800 hours on day

one of the estrous cycle. Forty eight hours later the animal was injected i.p. with 25 IU of human chorionic gonadotropin. Seventeen hours later the animal was euthanized by overdose of Phenobarbitol (Nembutal) and the oviducts were removed and flushed with PBS. The cumulus masses were collected and fixed in 4% paraformaldehyde in 0.1M PO_4 buffer for 1.5 hours at 4°C.

The tissue was then placed in JB4 Plus infiltration medium at 4°C for four hours, and then placed in number 0 gelatin capsules filled with JB4 Plus infiltration medium plus catalyst. The capsules were then centrifuged (Britton et al. 1991) for three minutes to force the tissue to the bottom of the gelatin capsules. The embedding medium was then allowed to polymerize overnight.

Thick sections (2μm) were cut on a Sorval Porter-Blum MT2-B ultramicrotome using a glass knife and placed on drops of water on glass microscope slides which were subsequently dried on a hot plate. Sections were then stained with toluidine blue and examined via standard light microscopy. Serial sections were examined until oocytes were visualized, at which time sections were cut, floated on water on glass slides and allowed to air dry overnight. These slides were then immunohistochemically stained following the standard staining technique previously described. Goat anti-hamster IgG diluted to 1:400 was used as the primary antibody and peroxidase-conjugated rabbit anti-goat IgG diluted at 1:20 was used as the secondary antibody.

Electron Microscopy

Ovaries were fixed in vivo by means of infusion via a Harvard infusion pump, all infusion being at the rate of 15.3 ml/minute via an 18 gauge cannula placed in the descending aorta or by cardiac puncture. Following incision of the right atrium, 50ml of 0.15M cacodylate buffer, pH 7.2, was infused to flush blood from the vessels. Immediately following the flushing, 150ml of cacodylate buffered fixative was infused. This fixative was a modification of that used by Doerr-Schott and Lichte (1986) such that the final concentrations of the components were 0.5% paraformaldehyde, 0.75% glutaraldehyde, and 0.06M cacodylate buffer. Upon completion of the infusion the ovaries were removed, sectioned into four pieces, and placed in fixative for 24 hours at 4°C.

The tissue was removed from the fixative, dehydrated in dimethylformamide (DMF), and prepared for embedding in lowicryl K4M (Electron Microscopy Sciences) using the following schedule (Altman, 1984):

50% DMF..... 20 minutes
75% DMF..... 20 minutes
90% DMF..... 20 minutes
2:1 DMF:lowicryl..... 60 minutes
1:1 DMF:lowicryl..... 60 minutes
100% lowicryl..... 24 hours at 4°C

Following the 24 hour infiltration, fresh lowicryl, mixed according to the manufacturers specifications, was used for tissue embedding. Polymerization of the lowicryl was carried out in a foil-lined Styrofoam chamber equipped with a black light (two 18" tubes, 25 watts, General

Electric) which emitted a wavelength of 360nm. The tissue was placed in number 0 gelatin capsules filled with lowicryl and suspended via thin wire at a lamp to tissue distance of 10 cm, and subjected to UV light for one hour at 4°C.

The tissue blocks were thick sectioned on a Sorval Porter-Blum MT2-B ultramicrotome using glass knives. These sections were placed on glass slides and stained with toluidine blue. Individual follicles were localized under the microscope and the blocks were trimmed to the area of a follicle for thin sections. Thin sections (gold to silver) were cut using a diamond knife and were transferred to 400 mesh nickel grids.

The grids were immersed in drops of 0.01M PBS with 5% ovalbumin for 10 minutes. The grids were then transferred without rinsing to drops of rabbit anti-hamster albumin diluted 1:400 with a solution of 50mM Tris HCl and 150mM NaCl at pH 7.4 where they were allowed to incubate overnight in a dark moist chamber at 4°C. The grids were then rinsed three times by placing them in drops of 0.01M PBS for ten minutes. Following blotting on lint-free paper the grids were transferred into drops of 15nm colloidal gold conjugated to protein A (Janssen) for 30 minutes. The colloidal gold was diluted at 1:20 with 1% ovalbumin in 0.02M PBS, pH 7.4. The grids were then rinsed three times in drops of 0.02M PBS at pH 7.4 for five minutes followed by a final rinse in distilled water for ten minutes. The grids were then counterstained by immersion in 1% uranyl acetate in 50% ethyl alcohol for 5.5 minutes, rinsing in distilled water and immersion in 0.018% lead citrate for 5.5 minutes and a final rinse in distilled water. Control tissue was treated with antibody substitution as described earlier.

Grids were viewed and photographed on a JEOL JEM 100S transmission electron microscope.

Injection of Labelled Immunoglobulin

Twenty mg samples of chromatographically purified Hamster IgG (Cappel) were reconstituted with 2ml of 0.1M Na_2HPO_4 (pH 8.5) and the pH was rapidly adjusted to 9.5 with 0.05M $NaCO_3$. Three tenths of a milligram of FITC was added and the pH again was rapidly adjusted to 9.5 with 0.05M $NaCO_3$. This mixture was allowed to react for one hour in the dark at room temperature. The solution was then separated by gel filtration through an 8ml column of Sephadex G-25 which had been swelled in physiological saline. Eight 1ml fractions were collected and analyzed in a Beckman DU-50 Spectrophotometer. Fractions were pooled such that a solution with a final concentration of 5mg/ml was obtained. Spectrophotometric analysis estimated an average FITC to IgG ratio of 3:1.

Injection of 1ml of FITC-conjugated hamster IgG solution (5mg/ml) was carried out via either the abdominal aorta or the femoral vein. One animal was injected at 1000 hours for each of the four days of the estrous cycle. The injection mass was allowed to circulate for one hour, at which time the ovaries were removed, sectioned into two pieces, and placed in 4% paraformaldehyde in 0.1M phosphate buffer for four hours. Control animals were injected i.v. with 1ml of FITC/physiological saline at the same FITC concentration (0.9μM) as the IgG/FITC solution. This injection was allowed to circulate for one hour before harvesting the ovaries. One animal from day three of the estrous cycle was injected with IgG/FITC and the circulation time was reduced to 30 minutes.

The tissue was removed from fixative and dehydrated using standard ethanol dehydration which was completed in two changes of 100% Hemo De (Fisher) for 30 minutes each. The tissue was then passed through a 50/50 mixture of Hemo De/Paraplast for 30 minutes and then through two changes of 100% Paraplast for 30 minutes each before final embedding in 100% Paraplast.

Paraplast sections were cut at 6μm, floated on a water bath of distilled water and gelatin, and mounted on glass slides. The sections were then deparaffinized using two changes of xylene for three minutes each and then rinsed with 100% ETOH. The slides were immediately coverslipped for fluorescence microscopy, as previously described, sealed with nail polish, and placed in the dark at 4°C for at least two hours before viewing on the Nikon Microphot FX Episcopic Fluorescent Microscope.

RESULTS

Folliclular development spans more than one estrous cycle and it is possible to find virtually any stage of follicular development at any time throughout the estrous cycle. In reviewing the ovary sections used in this study, follicles of virtually all stages of development, from primary unilaminar to antral follicles, were seen at every time period sampled. An exception was the occurrence of mature graffian follicles, which were visualized mainly in the later time periods (1800 and 2200 hours) on day four of the estrous cycle.

Indirect Staining Technique

Treatment of ovary sections with goat anti-hamster IgG (GAH-IgG) as a primary antibody followed with peroxidase or FITC-conjugated rabbit anti-goat IgG (RAG-IgG) as a secondary antibody resulted in labelling of the ZP and remnants of the liquor folliculi. Zonae pellucidae exhibit labelling in primary unilaminar follicles and continue throughout successive stages of development. The ZP of atretic follicles also label. The liquor folliculi did not label in many antral follicles, and in others labelling appears to be directed toward one side of the antrum.

Day one, 1000h

Follicles from this stage of the cycle exhibit intense labelling of the ZP when treated with GAH-IgG followed by FITC-conjugated RAG-IgG (Figs. 1 and 2). Control tissue (treated with FITC-conjugated RAG-IgG alone) did not exhibit labelling as seen in an ovary harvested at 1000h of day two (Fig. 4).

Day one, 1400h

Large follicles found in ovaries in this stage treated with GAH-IgG followed by FITC-conjugated RAG-IgG exhibit intense labelling of the ZP with a less intense labelling in the space between the granulosa cells (Fig. 3). High power magnification of ovary sections reveal a precise localization of the peroxidase reaction product over the ZP when treated with GAH-IgG followed by peroxidase-conjugated RAG-IgG (Figs. 5 and 7). Similar examination of control tissue (treated with peroxidase-conjugated RAG-IgG alone) exhibited a faint labelling over the area of the ZP (Figs. 6 and 8).

Day two, 0200h

Follicles at this stage of the estrous cycle exhibit labelling over the ZP when treated with GAH-IgG followed by peroxidase-conjugated RAG-IgG. This can be seen in primary unilaminar follicles as well as in multilaminar primary follicles (Fig. 9). Control tissue, consisting of sections through the same follicles exhibits only faint labelling when treated with peroxidase-conjugated RAG-IgG (Fig. 10).

Day two, 1000h

Labelling of the initial stages of ZP formation is seen in a primary unilaminar follicle in an ovary harvested at this stage of the estrous cycle when treated with GAH-IgG and peroxidase-conjugated RAG-IgG (Fig. 11). Multilaminar follicles in ovaries from this sample time exhibit labelling of the ZP when treated with GAH-IgG followed by either peroxidase or FITC-conjugated RAG-IgG (Figs. 13 and 14). Control tissue from this sample period did not exhibit labelling when treated with FITC-conjugated RAG-IgG alone (Fig. 4).

Day two, 1400h

Ovaries harvested at this stage of the estrous cycle reveal labelling of the ZP in unilaminar primary follicles (Fig. 12) and multilaminar primary follicles (Fig. 15) treated with GAH-IgG followed by peroxidase-conjugated RAG-IgG. Control tissue treated with peroxidase-conjugated RAG-IgG show faint labelling (Fig. 16). Tissue from this time period treated with goat anti-mouse IgA (GAM-IgA) followed with peroxidase-conjugated RAG-IgG show faint labelling of the ZP in follicles ranging from unilaminar primary to multilaminar primary (Figs. 17 and 18).

Day three, 1000h

Zona pellucida in multilaminar follicles in ovaries harvested at this stage of the estrous cycle exhibit intense labelling when treated with GAH-IgG followed with peroxidase-conjugated RAG-IgG (Figs. 19 and 20). Similar results were seen in follicles harvested at day three, 1400 hours (Figs. 21 and 22).

Day four, 1400h

Ovaries harvested at this stage of the estrous cycle contain multilaminar follicles with ZP which exhibit labelling when treated with GAH-IgG followed by FITC-conjugated RAG-IgG (Figs. 23 and 24).

Day four, 2200h

Figure 25 demonstrates labelling of a multilaminar follicle in an ovary treated with GAM-IgA followed with peroxidase-conjugated RAG-IgG. Similarly, the ZP of another multilaminar follicle labels intensely when treated with GAH-IgG and peroxidase-conjugated RAG-IgG (Fig. 26).

Immunohistochemical staining procedures conducted with antibodies to hamster IgG, FP, and albumin which were raised in rabbit instead of goat, yielded results similar to the above. Ovary sections treated with rabbit anti-hamster FP followed by peroxidase-conjugated goat anti-rabbit IgG exhibit labelling of ZP (Fig. 27) and liquor folliculi. Treatment of tissue with rabbit anti-hamster albumin followed by peroxidase-conjugated goat anti-rabbit IgG reveals intense labelling of the ZP (Fig. 28) and liquor folliculi.

ZP of oviductal oocytes and oocytes flushed from the oviduct exhibit labelling when treated with GAH-IgG followed by peroxidase-conjugated GAR-IgG.

Electron Microscopy

Using the protein A gold technique to label the ZP was successful with respect to albumin only. Tissue that was treated with rabbit anti-

hamster IgG as a primary antibody followed by treatment with Protein A gold conjugate with 15nm gold particles (PAG-15) did not yield positive results in any ovarian tissue type. The same holds true for tissue in which the primary antibody used was rabbit anti-hamster FP. However, when rabbit anti-hamster albumin is used as the primary antibody followed by treatment with PAG-15 there is a remarkably specific localization of the gold particles over the ZP (Fig. 29). Control tissue treated with PAG-15 only exhibited no labelling in the ovary (Fig. 30).

Fluorescein Conjugated Immunoglobulin Injection

To reiterate, fluorescein conjugated hamster IgG was injected i.v. into hamsters from each day of the estrous cycle. The injected IgG was allowed to circulate for specified periods of time, after which the tissue was collected and paraffin embedded as previously described. Subsequent tissue sections were viewed and photographed on a fluorescence microscope.

Day one, 1000h

Fluorescence studies of ovaries from this stage of the estrous cycle reveal that within one hour of injection, the IgG progressed to the level of the ZP (Figs. 31 and 32). Fluorescence is also seen in the liquor folliculi and in patches between granulosa cells (Figs. 33) presumably denoting initial formation of liquor folliculi. Antral follicles at this time period exhibit evidence of atresia (Fig. 34). Remnants of ZP seen at this stage of the estrous cycle do not exhibit fluorescence (Fig. 34) as is the case in

other stages of the cycle (Fig. 38). However, fluorescence is seen in ZP remnants which still enclose remnants of oocytes or macophages (Fig. 37).

Day two, 1000h

Examination of ovarian tissue from this stage of the estrous cycle reveal comparable results to those seen on day one. After one hour of circulation the IgG progressed to the level of the ZP in small bilaminar follicles (Fig. 35) as well as in multilaminar follicles. Patches of fluorescence are seen between granulosa cells of some multilaminar follicles (Figs. 36 and 37) presumably marking the initial formation of the liquor folliculi.

Follicles which exhibit labelling of the liquor folliculi at this time period also exhibit signs of atresia. Remnants of ZP of follicles from past cycles exhibit relatively little labelling (Fig. 38).

Day three, 1000h

Thirty minutes after injection of fluorescein labelled IgG at this stage of the estrous cycle the IgG advanced to the level of the ZP, however there appeared to be more fluorescence toward the outside of the ZP (Figs. 39 and 40). This suggests that thirty minutes may be near the lower limit of time required for circulating immunoglobulin to reach the ZP.

Fluorescein labelled IgG injected at this stage of the estrous cycle, when allowed to circulate for 1 hour exhibits localization in the ZP as well as in the liquor folliculi of developing antral follicles (Fig. 41) and larger antral follicles (Fig. 42).

Day four, 1000h

Ovaries harvested from animals injected at this stage of the estrous cycle demonstrate localization of fluorescein labelled IgG in the ZP (Fig. 43) and in the liquor folliculi of antral follicles.

Control tissue injected with fluorescein in physiological saline does not exhibit fluorescence outside of the blood vessels (Fig. 44).

DISCUSSION

The purpose of this study was to determine if the ZP functions as a barrier, limiting access of humoral immune agents to the oocyte. This is important in that the oocyte is capable of synthesizing "non-self" proteins and may be recognized as foreign by the immune system.

Since the oocyte develops in a manner similar to the spermatocyte, and the spermatocyte has been shown to produce proteins on its cell surface that are antigenic to the host immune system, one might assume that the oocyte also produces antigenic proteins specific to its cell coat. Such proteins would identify the oocyte as a foreign cell, therefore these cells must be protected against exposure to destructive elements of the immune system.

Immunohistochemical labelling techniques were used to determine the presence of IgG, FP, and albumin in the hamster ovary. In attempting immunohistochemical staining certain problems arise which should be considered before choosing a fixative, fixation method and antibody conjugate.

In choosing a fixative it is important to remember that fixation confers rigidity on tissue mainly by cross-linking proteins. The better the cross-linking the better the morphological preservation. It would thus seem that a fixative that causes a high degree of cross-linking would be preferable for histological preparations. Extensive cross-linking, however,

impairs antigens both by direct chemical effects and by structural distortion (Sternberger, 1986), thus a high degree of cross-linking is not preferable in tissue to be used for immunological studies.

A fixative that preserves immunoreactivity with one type of antibody may destroy the other epitopes of the same antigen. If one applies region-specific immunocytochemistry it becomes evident that different epitopes are distinct chemical individuals that react differently with different fixatives (Larsson, 1988). In other words, a fixative may preserve reactivity of one epitope in a certain antigen, but may completely destroy reactivity of other epitopes. Most fixatives bind covalently with amino groups and leave carboxy groups and tyrosine and histidine unaffected. In short, immunocytochemical detection of antigens in fixed tissue depends only on those groups that are not destroyed by the fixative (Sternberger, 1986). Fixation may also induce conformational changes in the antigens, thus total impairment of immunoreactivity may be extensive even though the fixative may directly affect only related chemical groups.

Fixation in itself may serve to extract or displace certain antigens. Many fixatives penetrate tissues quite slowly. If immersion fixation is to be used tissue blocks should be as small as possible otherwise the buffer used to dissolve the fixative may reach the tissue first and extract or displace the antigen before it is fixed (Larsson, 1988). Whenever possible fixation should be by perfusion rather than immersion.

In immunocytochemistry, use of poorly fixed tissue often yields spurious results. This is due to nonspecific binding and a high background generally attributed to Fc receptors or to "endogenous peroxidases." Red

blood cells and granulocytes possess a true endogenous peroxidase which becomes stained when exposed to diaminobenzidine and hydrogen peroxide (Sternberger, 1986). Poorly fixed areas in tissue as well as necrotic areas exhibit a tendency toward nonspecific staining even in the absence of primary antibody, and this is also referred to as "endogenous peroxidase" activity. This activity can be minimized by perfusion of the animal with physiological solutions followed by perfusion with fixative. The main purpose of the perfusion is not, however, elimination of red blood cells but assurance of better fixation than can be afforded by tissue immersion. Endogenous peroxidase activity can further be eliminated using various techniques such as treatment with methanolic hydrogen peroxide (Sternberger, 1986). A logical alternative for avoiding problems with endogenous peroxidases is to use staining procedures which do not employ diaminobenzidine or hydrogen peroxide, such as immunogold or immunofluorescence.

When using immunofluorescence one should consider using the mounting media described for coverslipping slides rather than using permount since permount has a tendency to fluoresce at various wavelengths. Also, this mounting media is preferable to a glycerin mounting procedure because the mounting media becomes a gel and provides better support and protection for the tissue, especially when viewed under high power objectives (100X) which may put pressure on the coverslip and inadvertently damage the tissue.

Advances in antibody production and purification now make it possible to produce large quantities of highly specific antibodies. This has

opened the door to the use of less mild fixatives and many optimal fixatives for tissue preservation have found application in immunocytochemistry.

Glutaraldehyde, by virtue of its cross-linking qualities, hardens tissues so much that it prevents its own penetration. Mixtures of formaldehyde and glutaraldehyde however, do not encounter this difficulty. Glutaraldehyde fixation may be followed by osmium tetroxide for further membrane stabilization, however if used in postembedding staining electron microscopy, the osmium must be removed by oxidation with hydrogen peroxide, periodic acid, or permanganate with oxalic acid.

Another consideration before attempting immunocytochemical staining is the location of the antigen. Antigens localized in extracellular spaces, as well as low molecular weight antigens are more prone to extraction or displacement than large molecular weight antigens (Larsson, 1988).

The tissue generated in this study performed well for light microscopic immunolabelling techniques, the only inconsistency being in the labelling of the liquor folliculi. The variation in labelling of the liquor folliculi of antral follicles may be related to the process of embedding the tissue. Since the liquor folliculi is a fluid, it, along with the other fluids of the tissue, is replaced by the embedding medium during the process of dehydration and infiltration. Thus it would be expected that much of the contents of these fluids would be extracted during tissue preparation.

Goat anti-mouse IgA was used as the primary antibody on randomly selected tissue to determine the presence of IgA in the ZP. The results indicate faint labelling which is due to cross-reactivity between mouse IgA and hamster IgA. Cross reactivity between hamster and other species,

including rodents, is expected to be minimal (John Coe, personal communication).

The faint labelling seen in controls treated with rabbit anti-goat IgG is attributable to cross-reactivity. This is expected since anti-goat IgG may cross-react with the hamster IgG of the ZP.

Labelling problems were encountered when the study shifted to electron microscopy. A factor of utmost importance in all immunocytochemistry is the binding of secondary antibody to the primary antibody. Protein A gold exhibits very weak binding when reacted with goat IgG (Amersham technical bulletin) thus poor results, if any, would be expected when using GAH-IgG as a primary antibody, as was used in the light microscopic study. Protein A gold exhibits strong binding when reacted with rabbit IgG (Amersham technical bulletin) thus an anti-hamster antibody raised in rabbit should give desirable results as a primary antibody followed by treatment with protein A gold.

When using anti-hamster IgG, anti-hamster FP, and anti-hamster albumin raised in rabbit followed with PAG-15, positive labelling was seen only on albumin treated sections. This would indicate that the anti-hamster IgG and anti-hamster FP did not bind to the sections. Since this tissue differs from that used in the light microscopic study in two ways, fixative used and embedding media, the explanation for the lack of labelling of FP and IgG may lie in these two areas.

As previously described, a particular fixative may provide for excellent staining of one antigen while completely destroying other antigens. A paraformaldehyde/glutaraldehyde mixture was used for fixation of tissues to be examined by electron microscopy. In this case

glutaraldehyde could be implicated as the undesirable fixative since binding of the primary antibody to paraformaldehyde fixed tissues did not appear to be a problem in the light microscopic study. The use of gluteraldehyde, however, was necessary to obtain morphology suitable for electron microscopic examination.

The other variable that was changed between the light and electron microscopic studies was the use of lowicryl K4M and its accompanying preparation for embedding in place of JB4+. Preparation for embedding in JB4+ involved tissue immersion in JB4+ infiltration medium as a dehydration procedure whereas lowicryl embedding required tissue dehydration prior to immersion in the lowicryl. Dimethylformamide was used as the dehydration medium as previously discussed.

In preliminary experiments for light microscopy, DMF was used as a dehydrating agent prior to embedding in JB4+. No morphological advantage was seen in tissue prepared in this manner, so the DMF was dropped from the protocol. Immunolabelling of the ZP was observed in these DMF dehydrated tissues, this would seem to indicate that DMF dehydration is compatible with the antigens in question and quite probably is not responsible for the failure to label tissues for electron microscopy.

Lowicryl is generally accepted as the polymer of choice when working with immunochemistry at the electron microscopic level. One of the advantages of lowicryl being that it can be used at low temperatures which protects tissues and presumably antigen binding sites. However, some antigens that have been detected using immunogold reagents on ultrathin frozen sections were undetectable when embedded in lowicryl K4M (Kerjaschki et al., 1986). No concrete explanation has been given

for this phenomena but one possible explanation is that the ultraviolet light used for polymerization of the lowicryl may have adverse effects on some antigens.

The results of both the light and electron microscopic studies indicate that intra-ovarian albumin is localized in the ZP and liquor folliculi of hamster ovarian follicles. Albumin has a negative charge, thus it is not surprising to find it bound to the ZP since, according to Sellens and Jenkinson (1975), the ZP might be expected to bind negatively charged molecules. Albumin is also described as a carrier molecule which is responsible for mediating functions of steroid-producing and steroid-responding cells (Krishna and Spanel-Borowski, 1989). Albumin is also implicated in initiating endocytosis of a ligand with or without internalization of the albumin itself, as well as being responsible for removing ligands from target cells. The possibility then exists for albumin to carry substances to the oocyte and at the same time remove proteins from the oocyte.

The localization of anti-hamster IgG in the ZP and not in the oocyte suggests that the ZP is impermeable to hamster IgG. It is not known if IgG is bound to the ZP or if it is merely trapped within the fibrillogranular matrix of the ZP. Since IgG is an initiator of the complement fixation pathway it would seem imperative to the survival of the oocyte that it not be allowed to reach the oocyte where it could literally mark the oocyte as a target for cell lysis and phagocytosis.

Anti-hamster FP also localized in the area of the ZP but not within the oocyte. This suggests that the ZP of the hamster is impermeable to FP. The nature of binding of the FP to the ZP is not known and the possibility

that FP is trapped in the ZP rather than bound to it must be considered. To reiterate, FP is the hamster homologue to serum amyloid protein and/or C-reactive protein from other mammals and has been implicated in the initiation of the complement fixation pathway and in enhanced phagocytosis. Again, a barrier to prevent this protein from reaching the oocyte is imperative.

The liquor folliculi is a transudate of blood serum, and as such contains many of the same proteins. This means that serum proteins must traverse the endothelial wall of the thecal capillaries and pass either through or between granulosa cells to reach the ZP and to eventually form liquor folliculi in the antral cavity of the follicle.

The injection studies conducted with fluorescein conjugated hamster IgG demonstrate that circulating IgG reaches the level of the ZP in less than 30 minutes. These also reveal that the immunoglobulin is located between granulosa cells, which is in agreement with findings of the indirect immunolabelling study. Large patches of fluorescence located between the granulosa cells are believed to be the initial stages of development of the liquor folliculi and thus depicts the initial formation of the antrum of the follicle.

Control animals, injected with FITC in physiologic saline, did not exhibit labelling outside of the blood vessels. This indicates that the vessels of the theca are not readily permeable to FITC that has been conjugated to physiological saline, but are permeable to FITC-conjugated IgG. This suggests that a mechanism exists for allowing passage of certain molecules while impeding the passage of others. In the case of IgG this process may be receptor mediated as previously described.

Immunoglobulin G is the only class of immunoglobulin that is known to cross the placental barrier and this phenomena is believed to be a receptor mediated event (Goodman, 1987; Steward, 1974). Immunoglobulin G Fc receptors have been isolated and it is believed that these receptors play a role in the transport of IgG (Van de Winkel and Anderson, 1991).

Similar studies using fluorescein conjugated serum globulins were conducted in the rat by Mancini et al. (1963). They describe patches of fluorescence in the oocyte itself. They concluded that the ZP was at least partially permeable to serum globulins. The findings of the current study do not support their results. No fluorescence, beyond that seen in control animals, was seen in the oocytes. Thus the suggestion that the ZP is impermeable to serum IgG is supported.

At this point a brief summary of events seems appropriate. The ZP and liquor folliculi both label for the presence of IgG, FP, and albumin. These proteins are derived from serum and become localized in the ZP and antrum.

Since both IgG and FP are known to initiate the complement fixation pathway, and all the necessary complement components have been found in the liquor folliculi, it seems that a system exists which could cause cell death on a large scale. This system would also allow for subsequent phagocytosis of the tissue. This allows for the rather rapid and complete follicular atresia that is seen in hamsters.

The function of an oocyte is to mature to the stage of ovulation, become fertilized, and develop into a viable embryo. If the follicular oocyte is surrounded by proteins, the primary function of which is complement fixation, then a mechanism must exist which impedes the

initiation of the complement cascade. Not all follicles mature to ovulation. Some become atretic and their cells, including the oocyte, are phagocytized. This study raises the possibility that atresia is due to an inability on the part of the oocyte to block the complement cascade in the follicle. This is evidenced by the fact that remnants of zona pellucida atretica seen in the injection study did not exhibit the fluorescence afforded by the FITC-conjugated hamster IgG unless remnants of the oocyte or phagoctyes were seen inside the borders of the ZP. However ZP remnants seen in the indirect labelling study did exhibit labelling. This suggests that IgG is not circulated to zona pellucida atretica that do not contain antigenic substances.

It has been suggested by Chamberlin et al. (1989) that some proteins of the ZP are only coded for by the oocyte genome. The possibility exists that one or more of these proteins may function to block the initiation of the complement fixation pathway. In order to do this, these proteins would themselves have to localize in the ZP. This suggests a possible role for albumin in the ZP, since, as previously described, albumin may be responsible for extraction of a ligand from the target cell. The albumin may be responsible for either the extraction of complement blocking proteins from the oocyte or sequestering these proteins in the ZP, or perhaps both.

This study suggests that all ovarian follicles are viewed equally by the immune system and that only follicles containing oocytes capable of producing a complement blocking protein will mature to ovulation. Oocytes of atretic follicles perhaps lack the ability to synthesize the proteins required to block the complement pathway. This may be brought

about by chromosomal abnormalities that may have occurred during the long arrested prophase of meiosis I.

In summary, the ZP of the hamster has been found to impede the entry of IgG, FP,and albumin into the oocyte, thereby providing a barrier between the oocyte and the maternal immune system. In addition, the ZP holds proteins associated with the complement fixation pathway. Under the appropriate conditions the complement system is blocked and the oocyte can proceed to ovulation. The suggestion is made that the oocyte is responsible for its own survival by producing proteins that block the complement cascade. Oocytes which are not capable of providing for their own protection become atretic and are phagocytized.

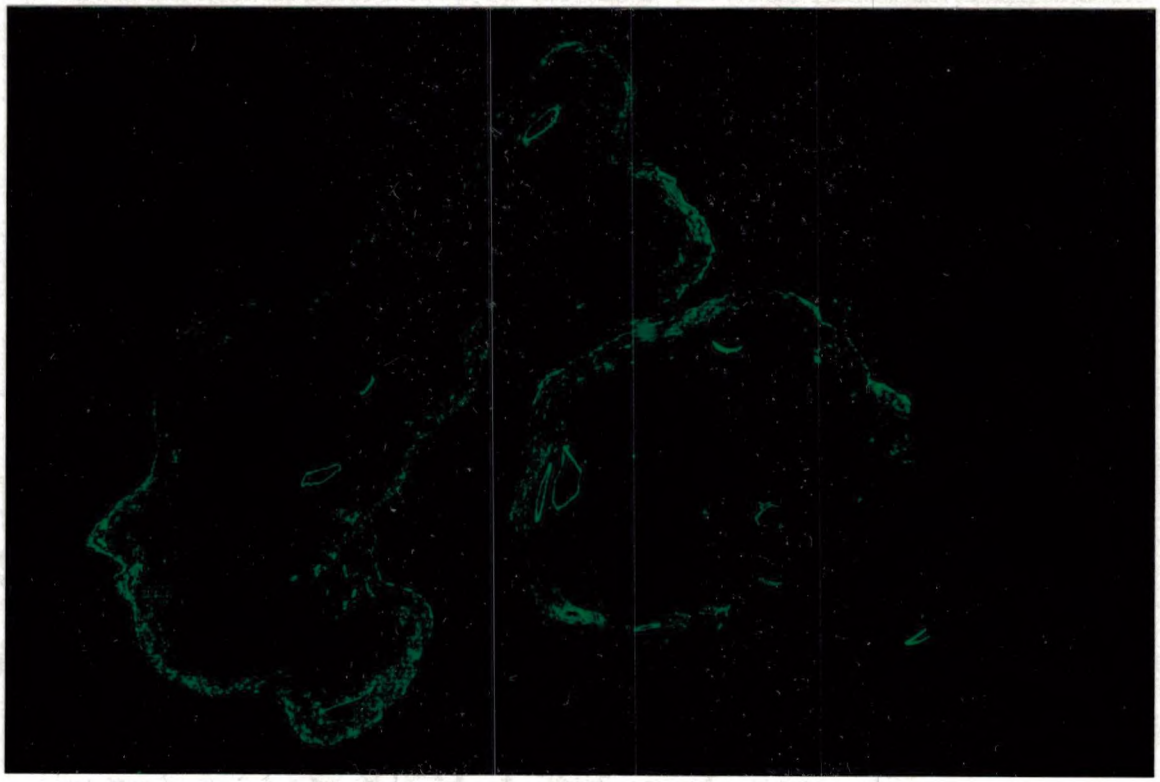
APPENDICES

Appendix A Plates And Figures

PLATE I

Figure 1. Fluorescence micrograph of an ovary harvested at 1000 hours on day one of the estrous cycle. Treated with GAH-IgG and FITC-conjugated rabbit anti-goat IgG as a secondary antibody. X 180.

Figure 2. Fluorescence micrograph of a hamster ovary harvested at 1400 hours on day one of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody and FITC-conjugated rabbit anti-goat IgG as a secondary antibody. Note the fluorescence between the granulosa cells in the large follicle (right). X 460.



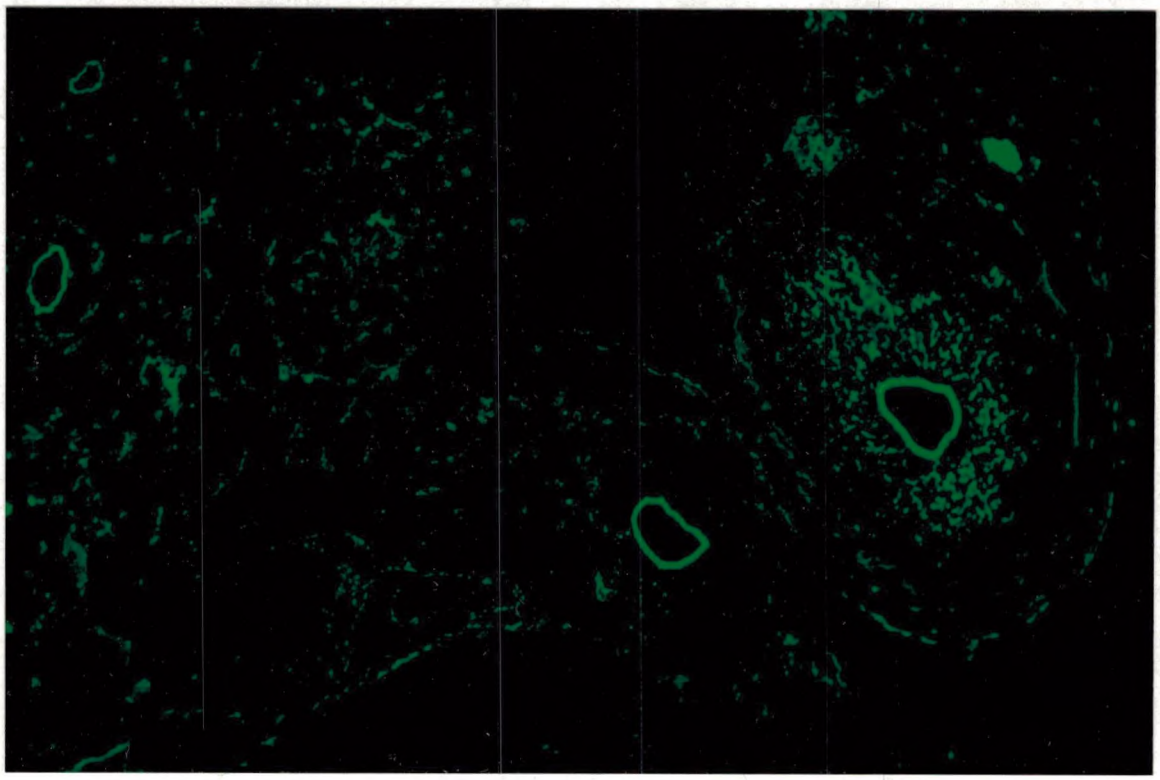
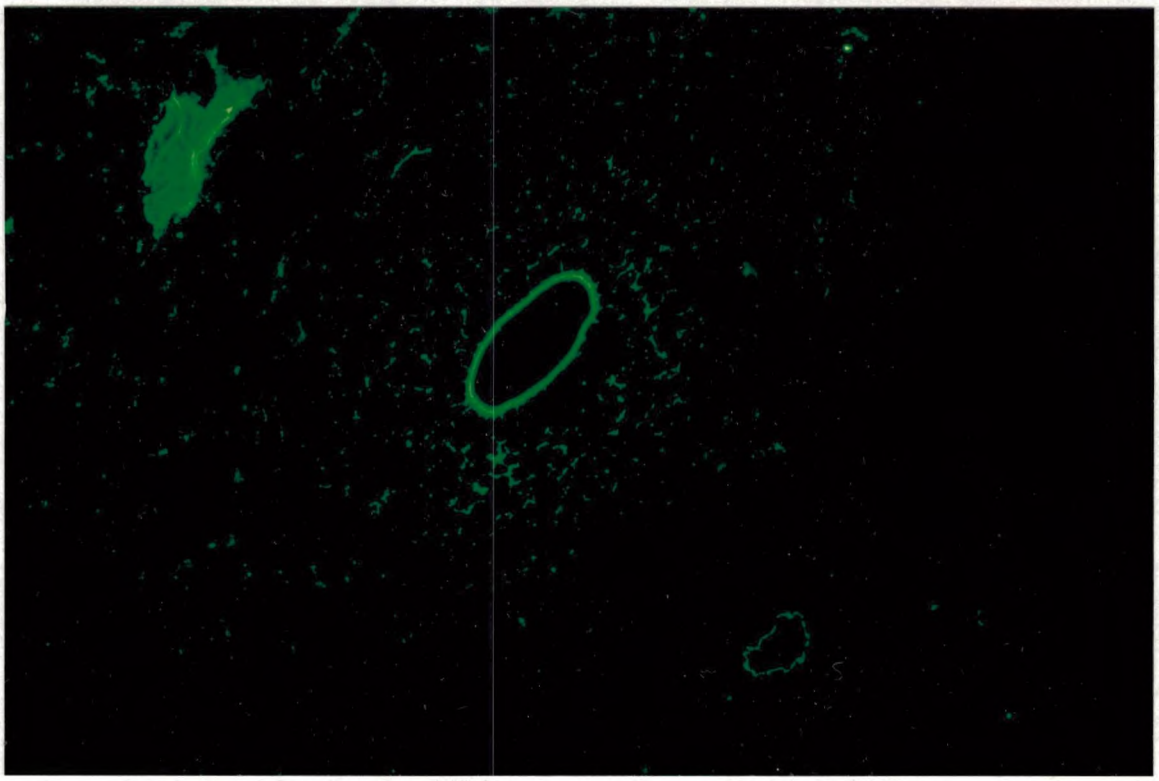


PLATE II

Figure 3. Fluorescence micrograph of an ovary harvested at 1400 hours on day one of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by FITC-conjugated rabbit anti-goat IgG as a secondary antibody. Note the slight labelling between the granulosa cells as well as the labelling of remnants of zona pellucida atretica (upper left). X 900.

Figure 4. Fluorescence micrograph of an ovary harvested at 1000 hours on day two of the estrous cycle. Control tissue treated with FITC-conjugated rabbit anti-goat IgG only. X 900.



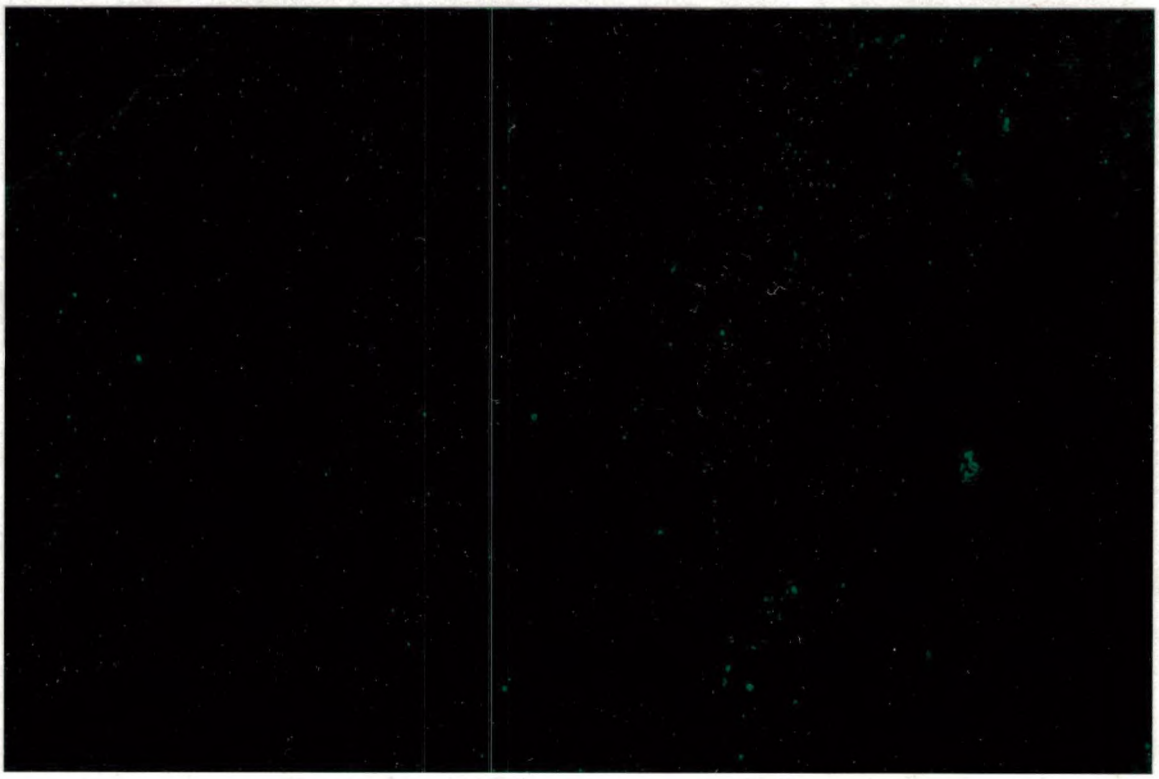
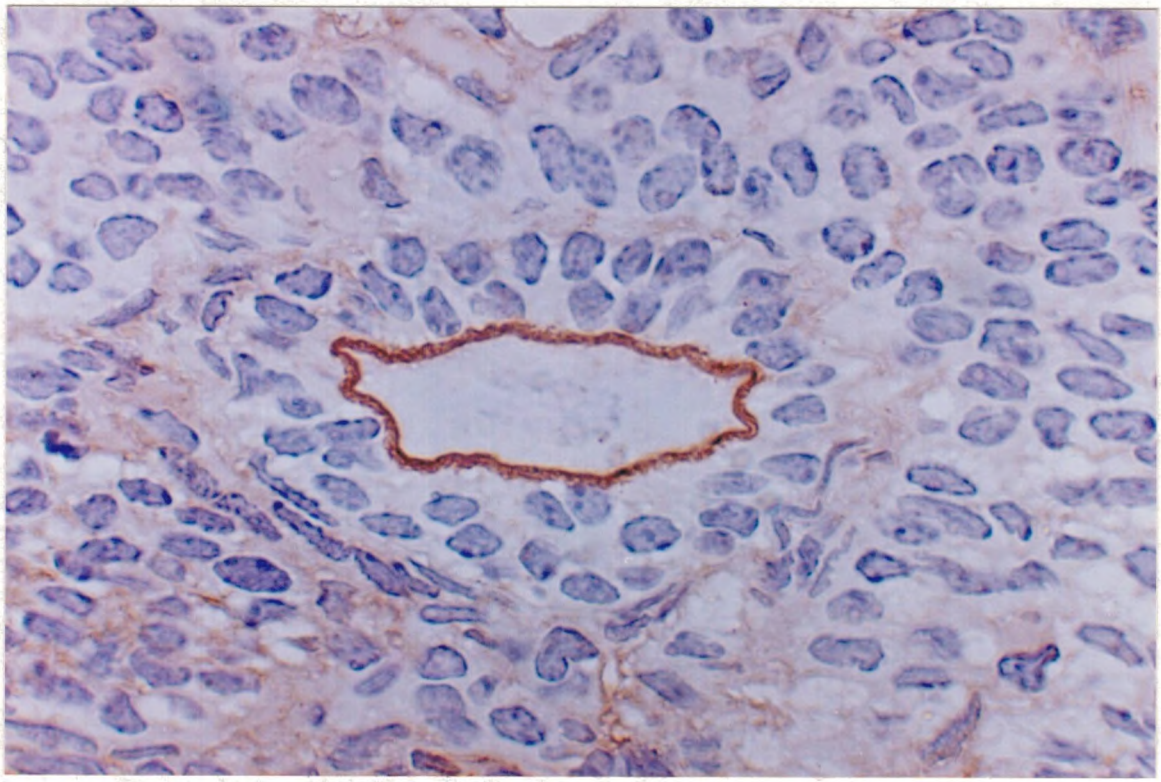


PLATE III

Figure 5. Light micrograph of an ovarian follicle in an hamster ovary harvested at 1400 hours on day one of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 4600.

Figure 6. Light micrograph of an ovarian follicle in an hamster ovary harvested at 1400 hours on day one of the estrous cycle. Treated with peroxidase-conjugated rabbit anti-goat IgG only. X 4600.



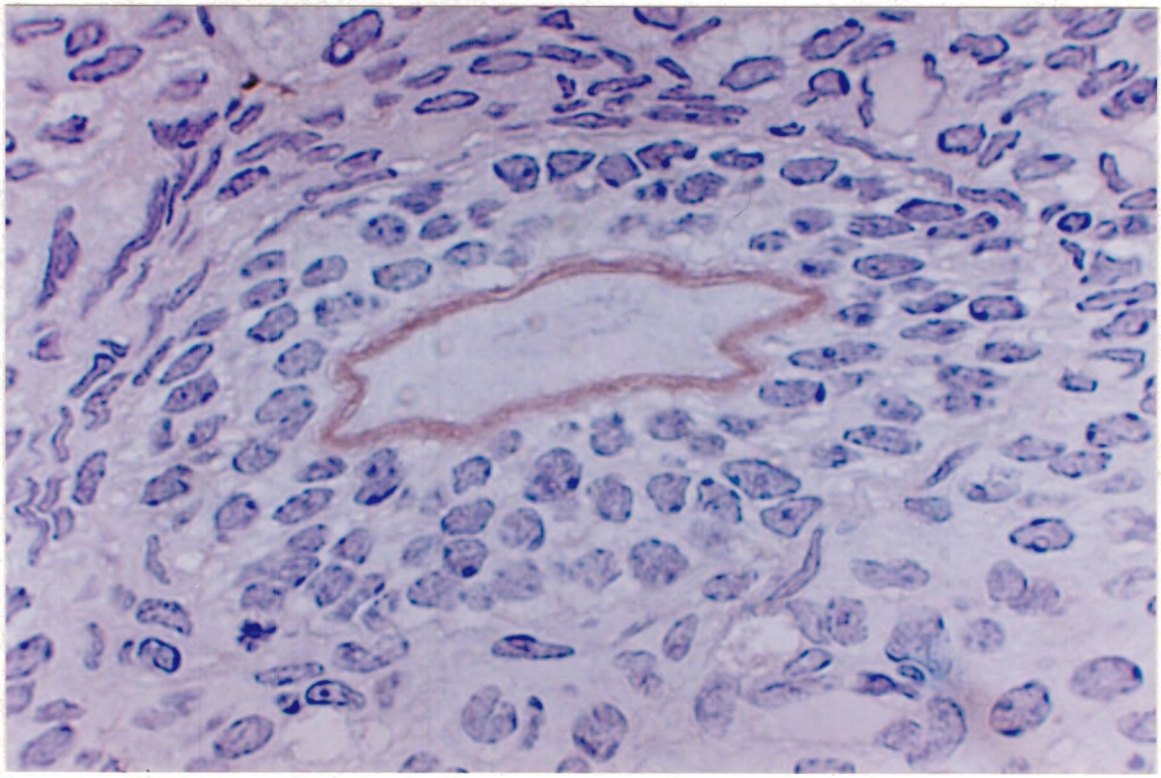
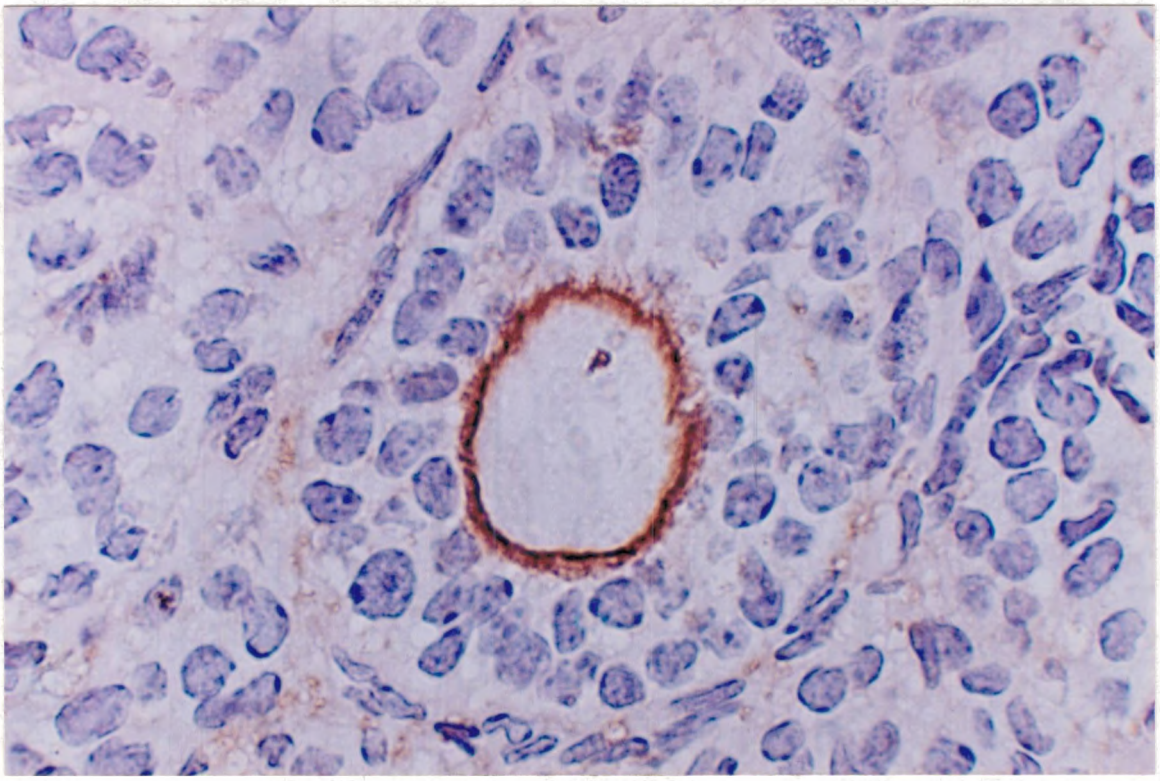


PLATE IV

Figure 7. Light micrograph of a hamster ovary harvested at 1400 hours on day one of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 4600.

Figure 8. Light micrograph of a hamster ovary harvested at 1400 hours on day one of the estrous cycle. Treated with peroxidase-conjugated rabbit anti-goat IgG only. X 1800.



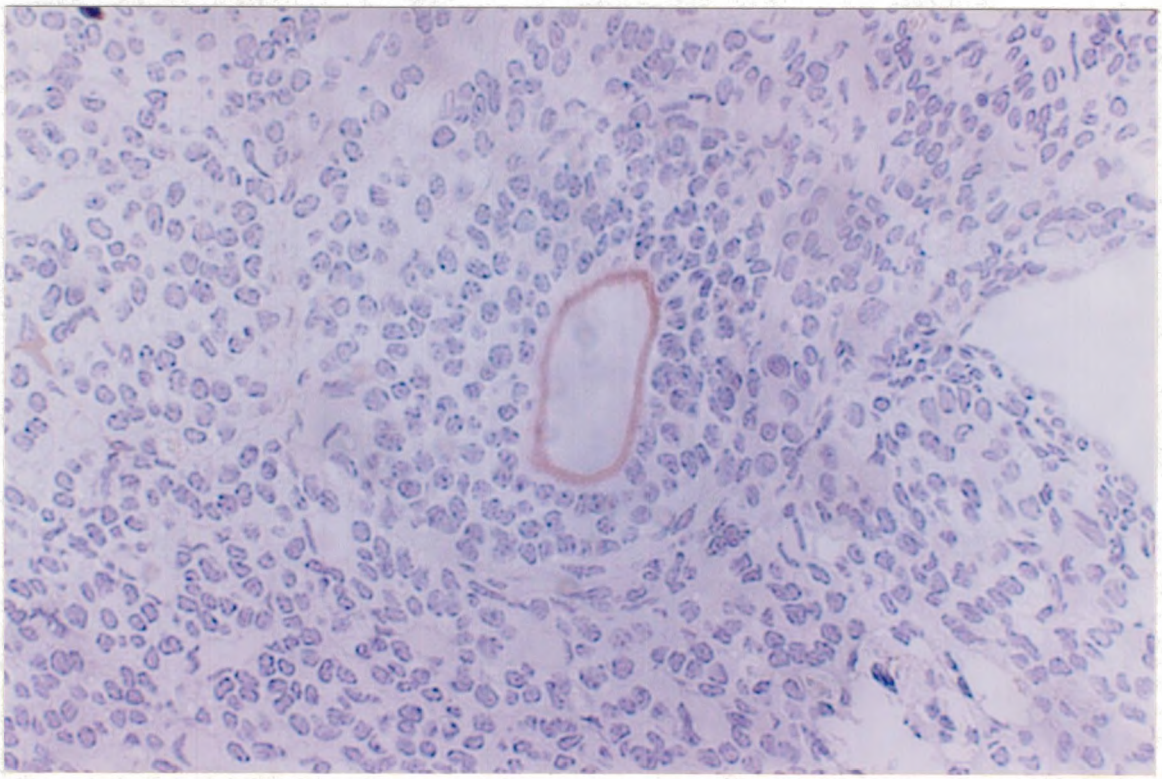
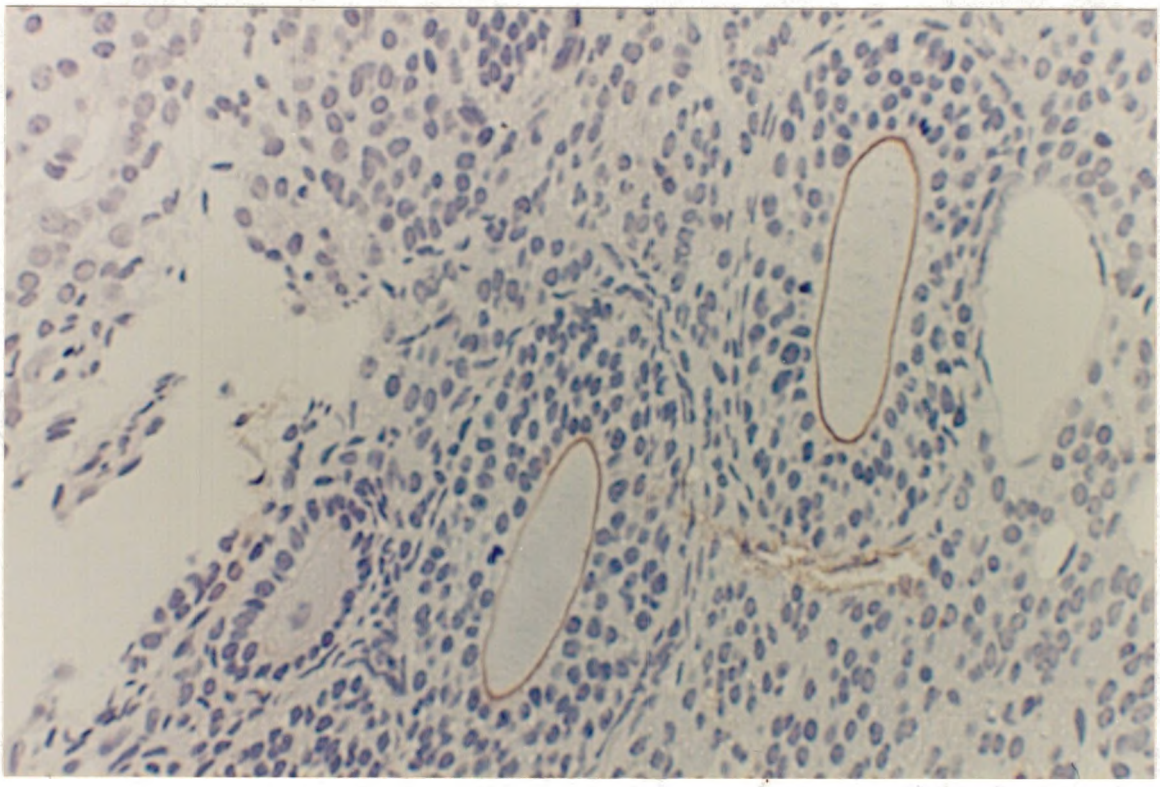


PLATE V

Figure 9. Light micrograph of three follicles located in an ovary harvested at 0200 hours on day two of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. Note the faint labelling in the primary unilaminar follicle. X 1800.

Figure 10. Light micrograph of section taken through the same follicles seen in Fig. 9. Control tissue treated with peroxidase-conjugated rabbit anti-goat IgG only. X 1800.



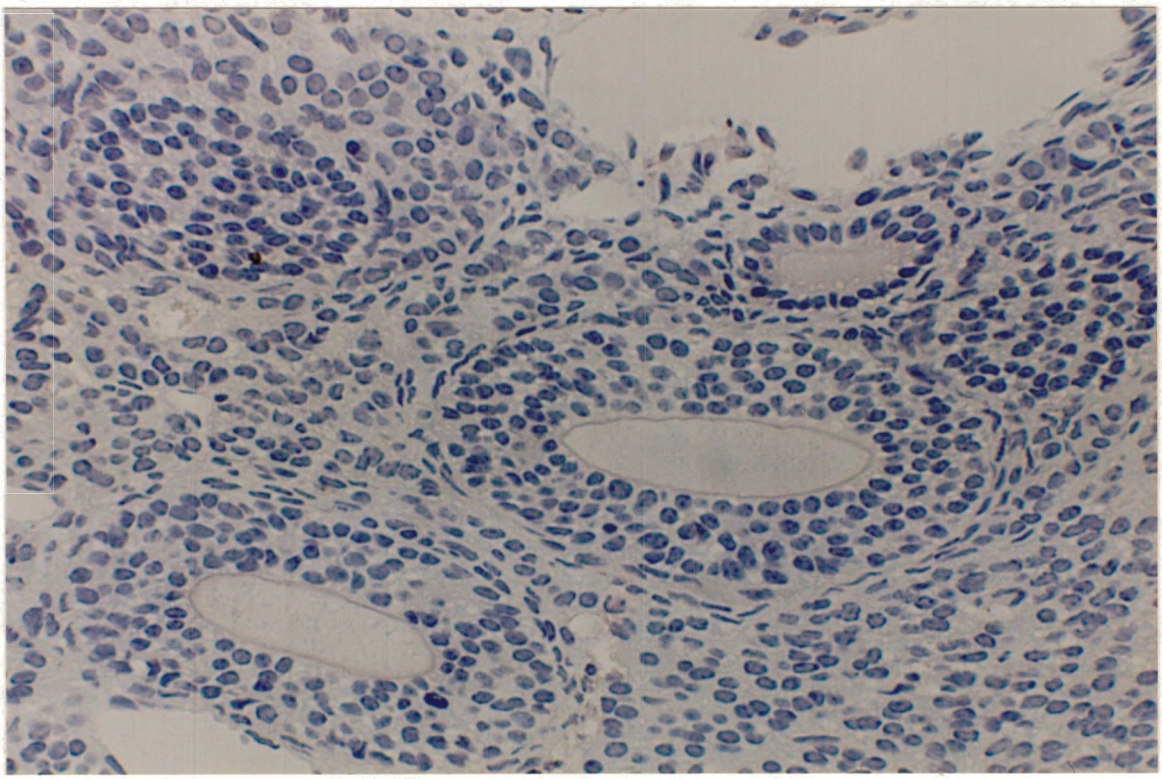
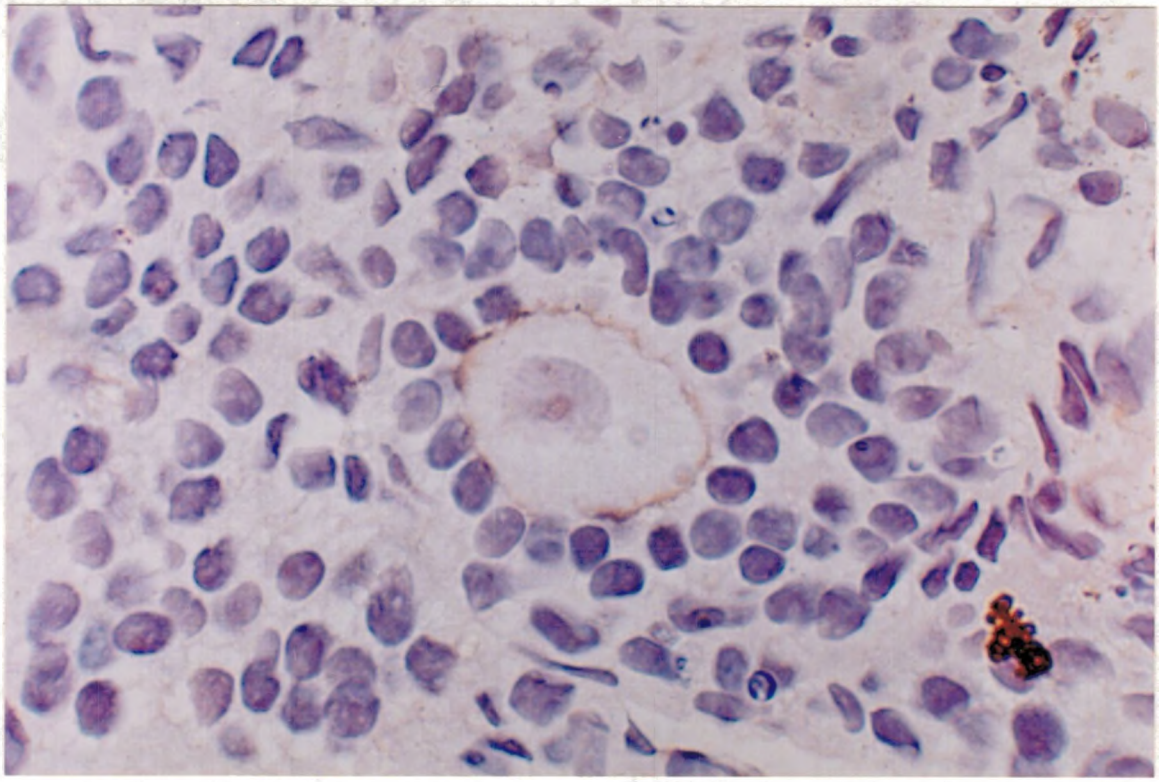


PLATE VI

Figure 11. Light micrograph of a primary unilaminar follicle seen in an ovary harvested at 1000 hours on day two of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 4600.

Figure 12. Light micrograph of a primary unilaminar follicle in an ovary harvested at 1400 hours on day two of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 4600.



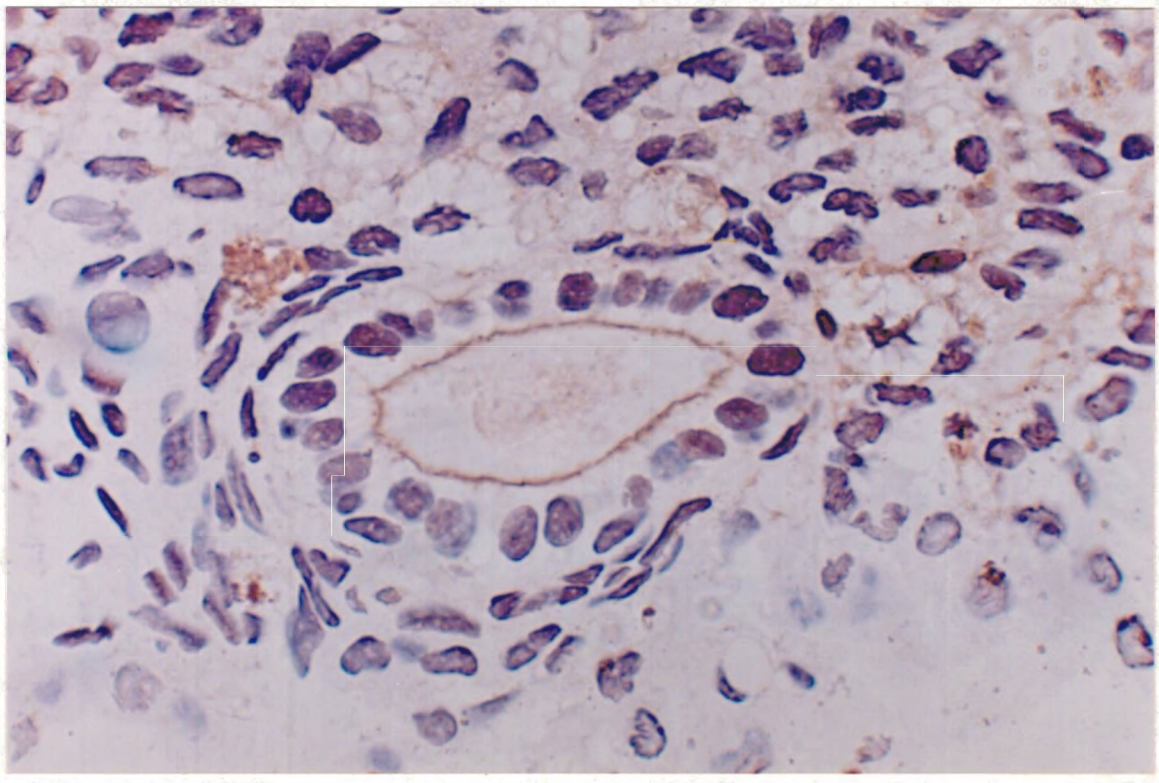
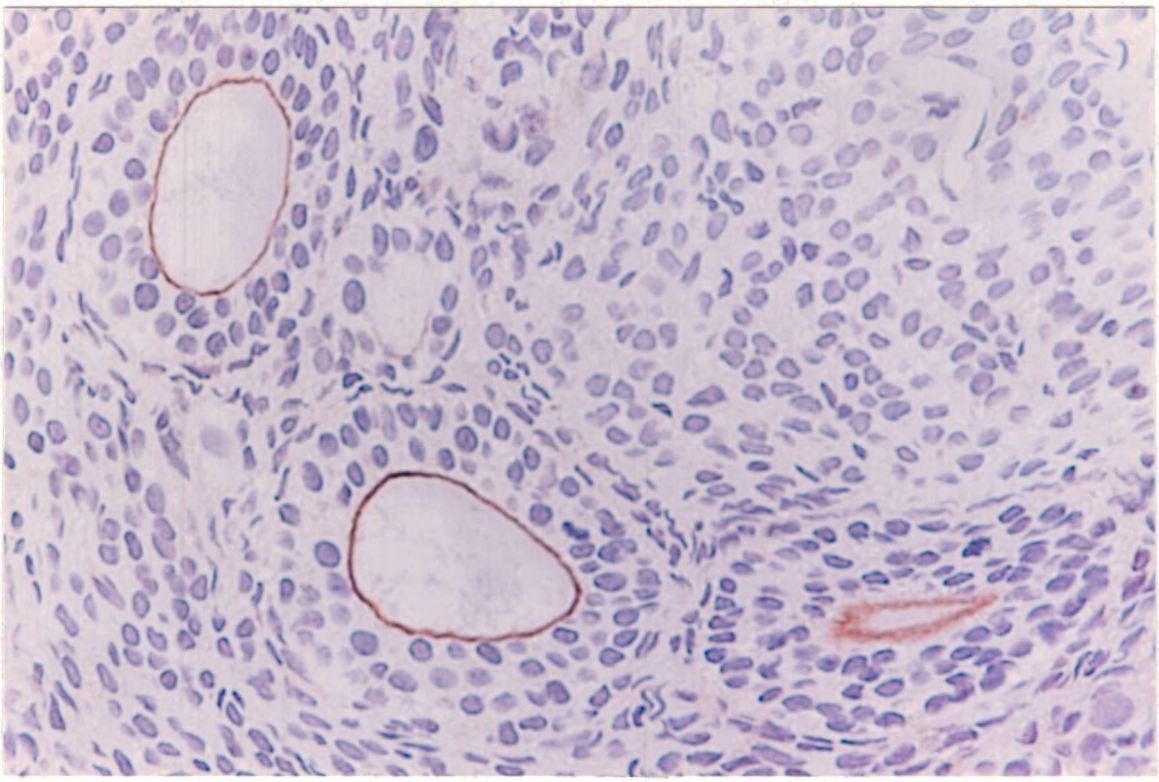


PLATE VII

Figure 13. Light micrograph of an ovary harvested at 1000 hours on day two of the estrous cycle. Treated with Goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.

Figure 14. Fluorescence micrograph of a section through the same follicles seen in Fig. 13. Treated with goat anti-hamster IgG as a primary antibody followed by FITC-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.



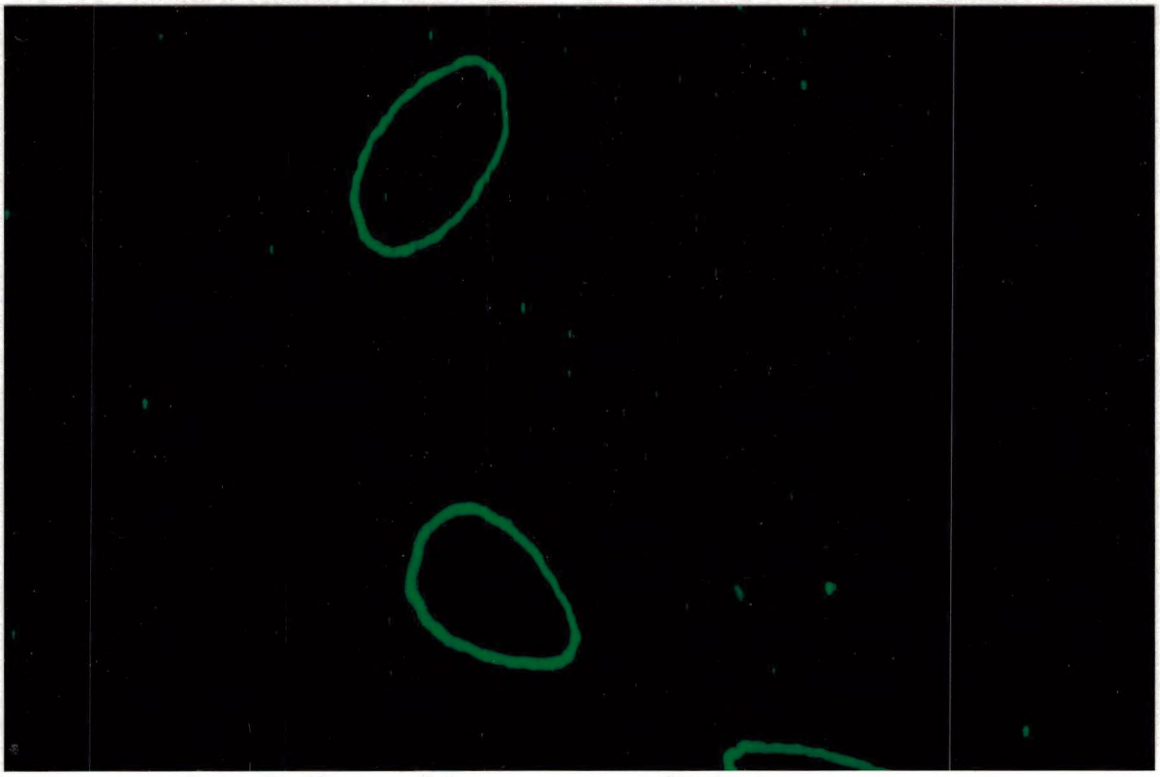
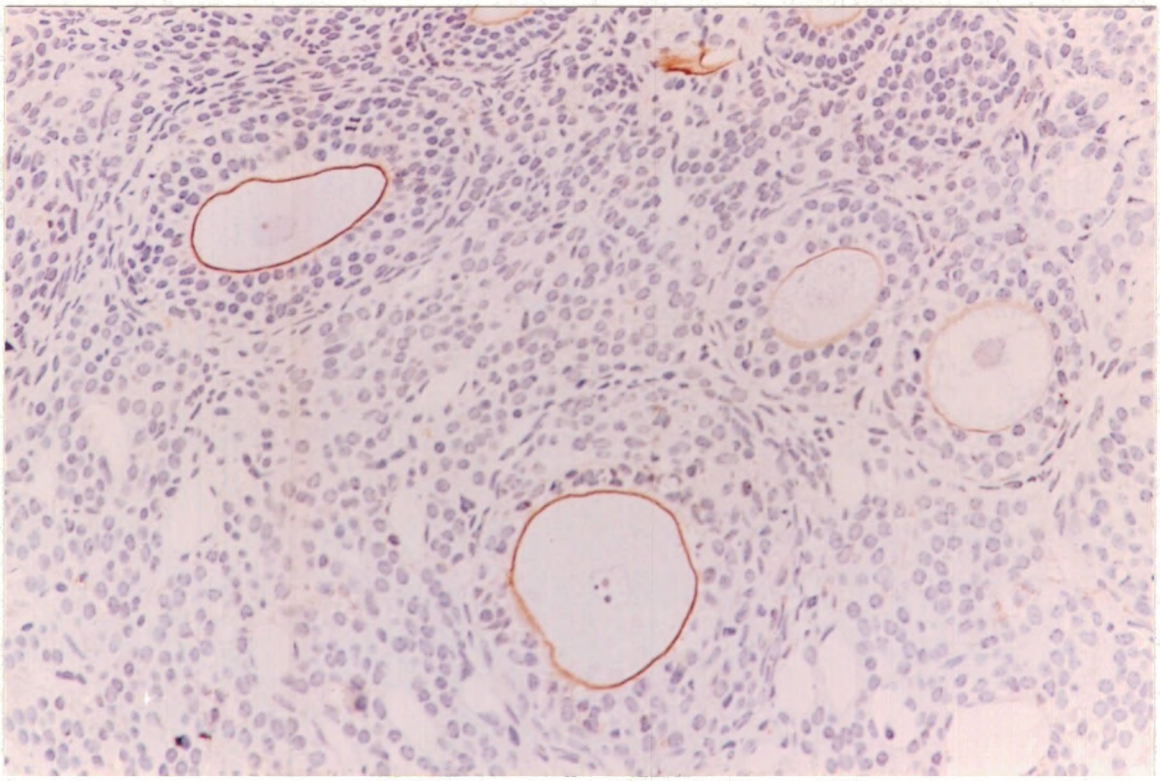


PLATE VIII

Figure 15. Light micrograph of an ovary harvested at 1400 hours on day two of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 900.

Figure 16. Light micrograph of sections through the same follicles seen in Fig. 15. Control tissue treated with peroxidase-conjugated rabbit anti-goat IgG only. X 900.



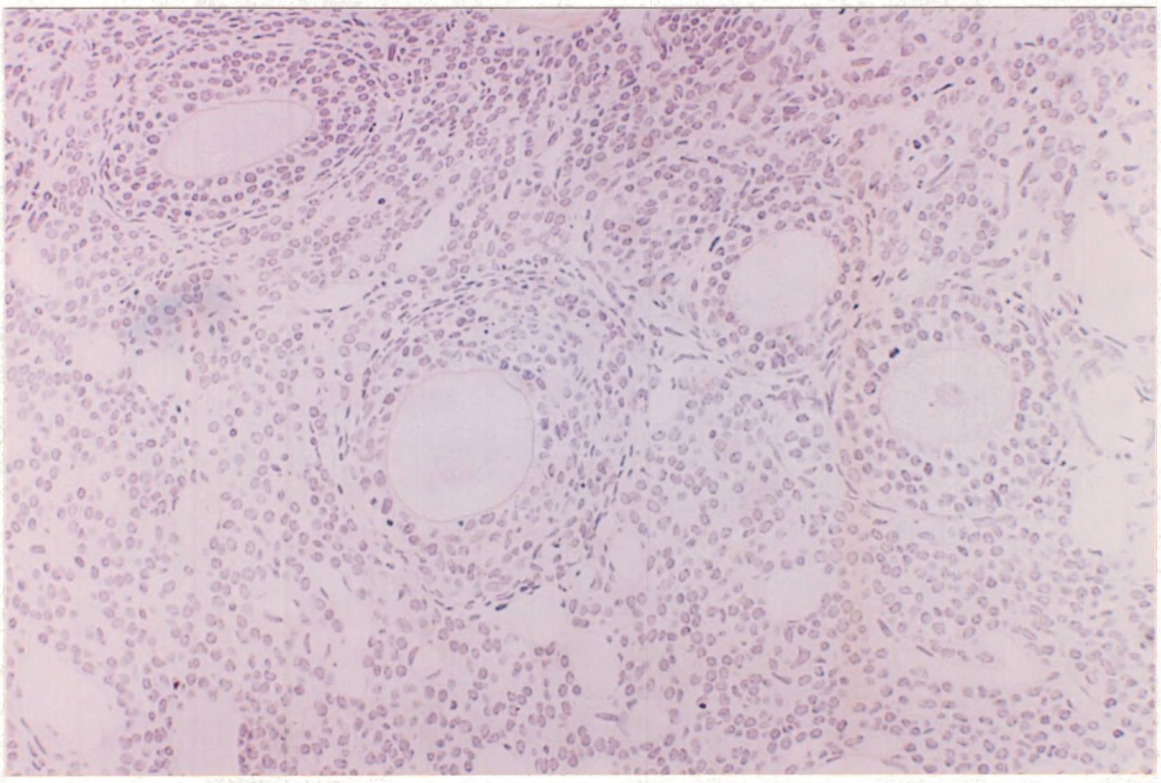
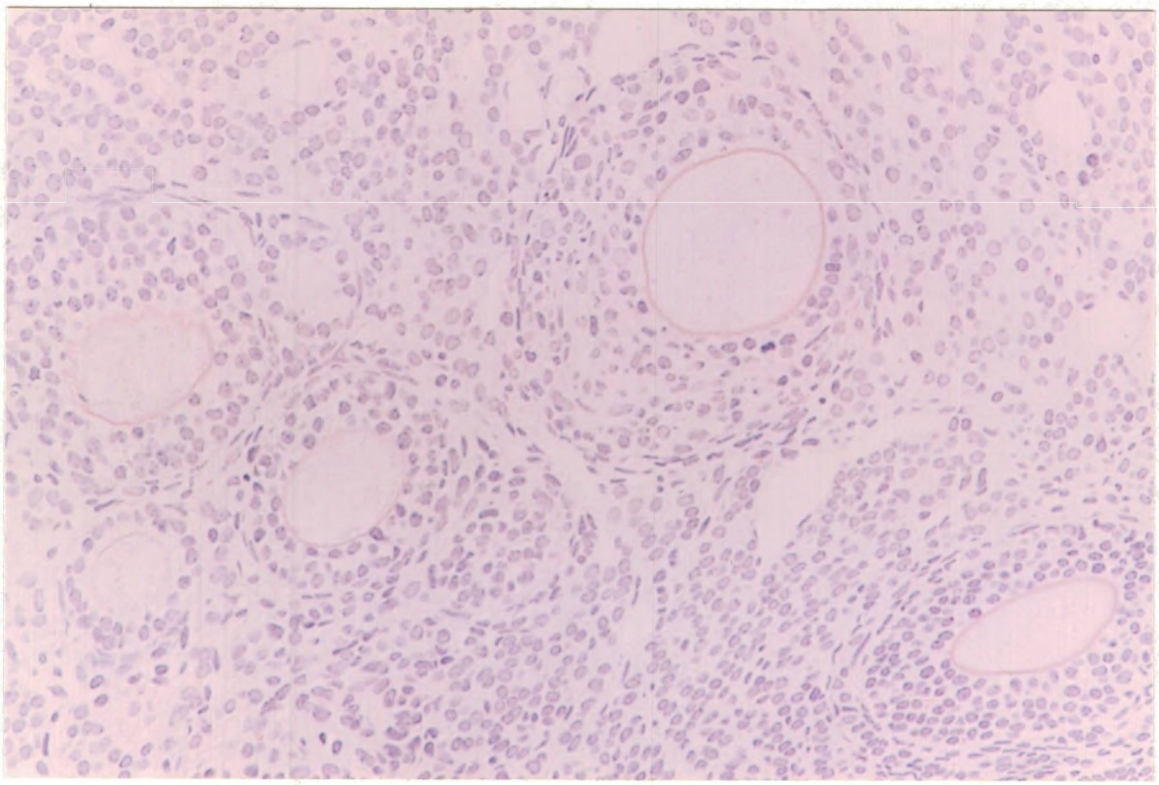


PLATE IX

Figure 17. Light micrograph of an ovary harvested at 1400 hours on day two of the estrous cycle. Treated with goat anti-mouse IgA as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 900.

Figure 18. Light micrograph of two multilaminar follicles in an ovary harvested at 1400 hours on day two of the estrous cycle. Treated with goat anti-mouse IgA as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.



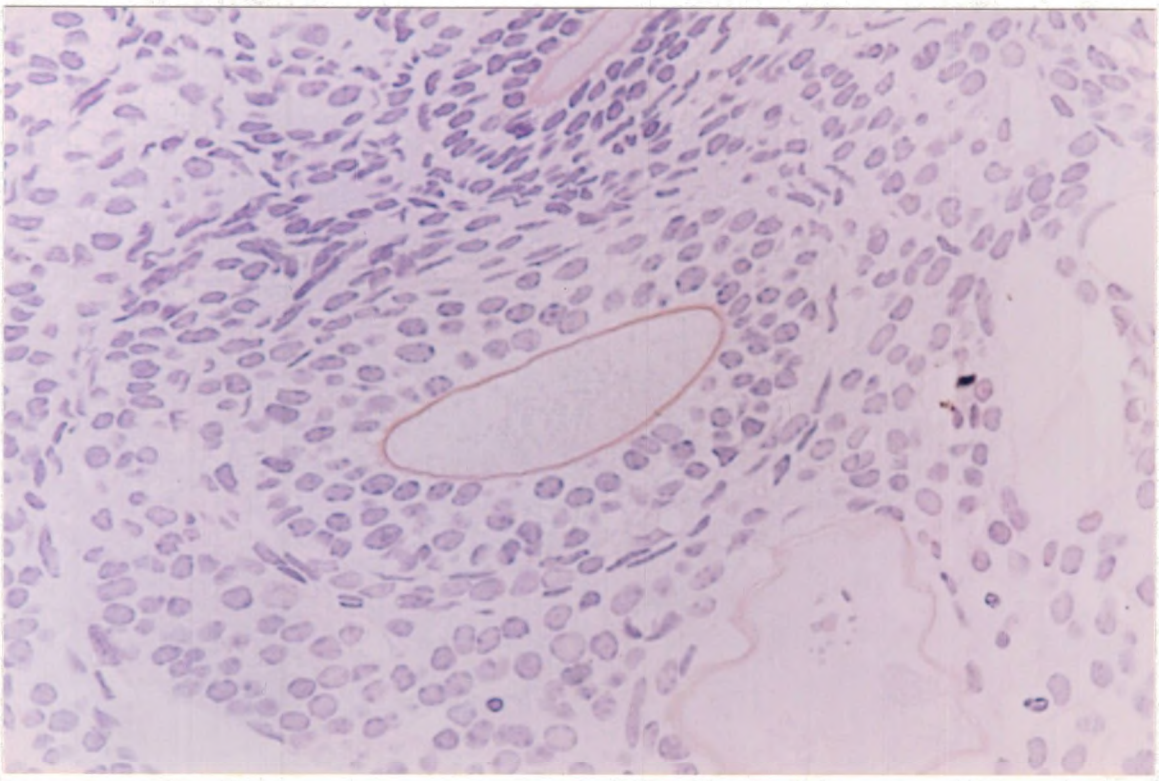
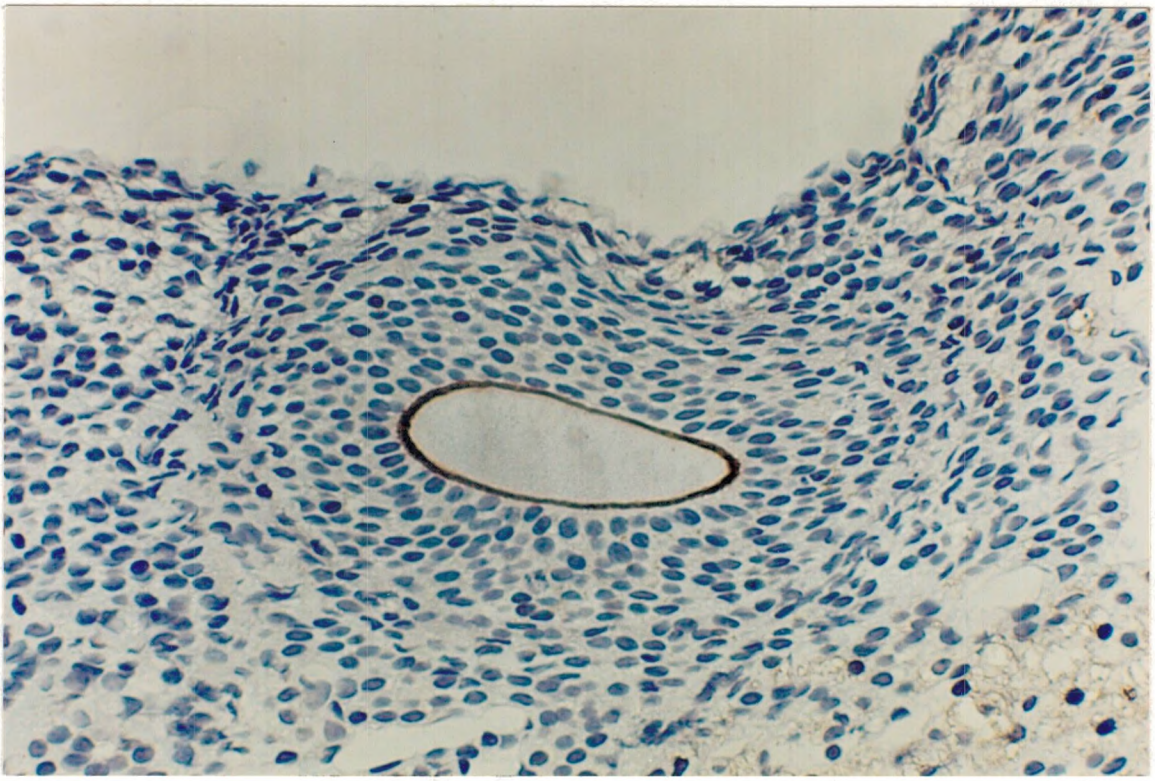


PLATE X

Figure 19. Light micrograph of a multilaminar follicle in an ovary harvested at 1000 hours on day three of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.

Figure 20. Light micrograph of two multilaminar follicles seen in an ovary harvested at 1000 hours on day three of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.



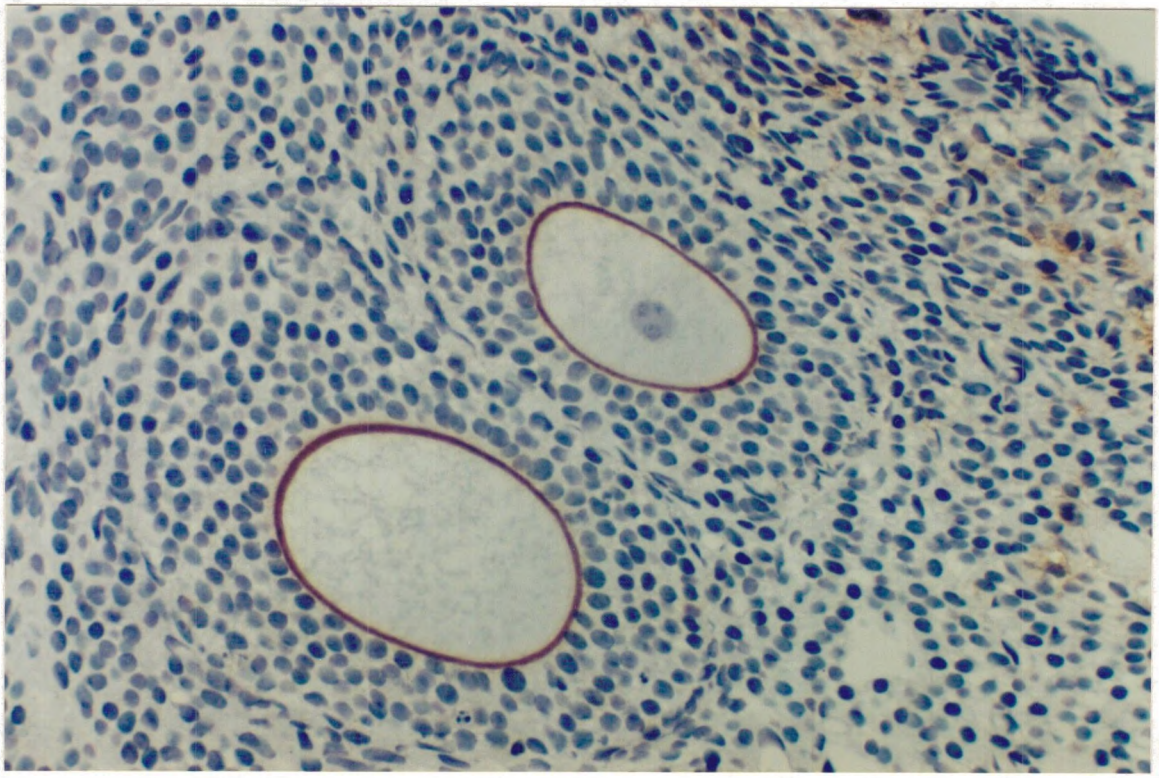
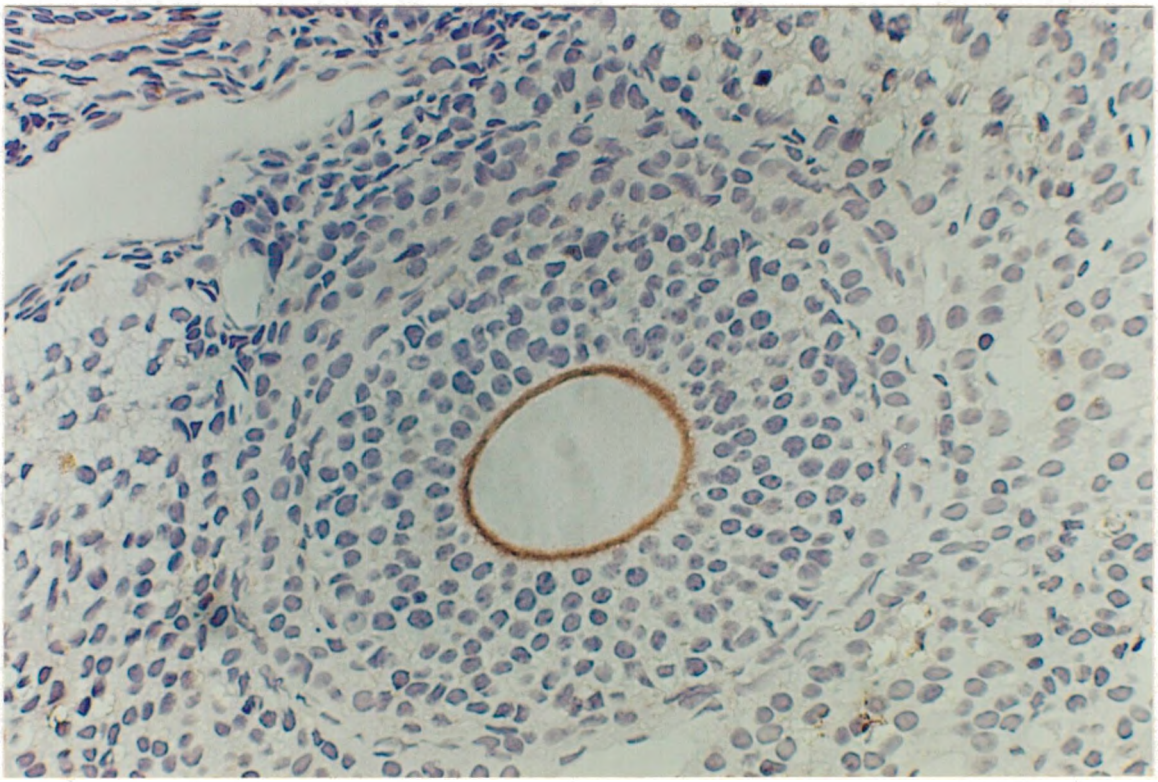


PLATE XI

Figure 21. Light micrograph of a multilaminar follicle seen in an ovary harvested at 1400 hours on day three of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.

Figure 22. Light micrograph of an ovary harvested at 1400 hours on day three of the estrous cycle. Treated with Goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. Note the labelling in the small bilaminar follicle (center) as well as the labelling of the remnants of the zona pellucida atretica. X 1800.



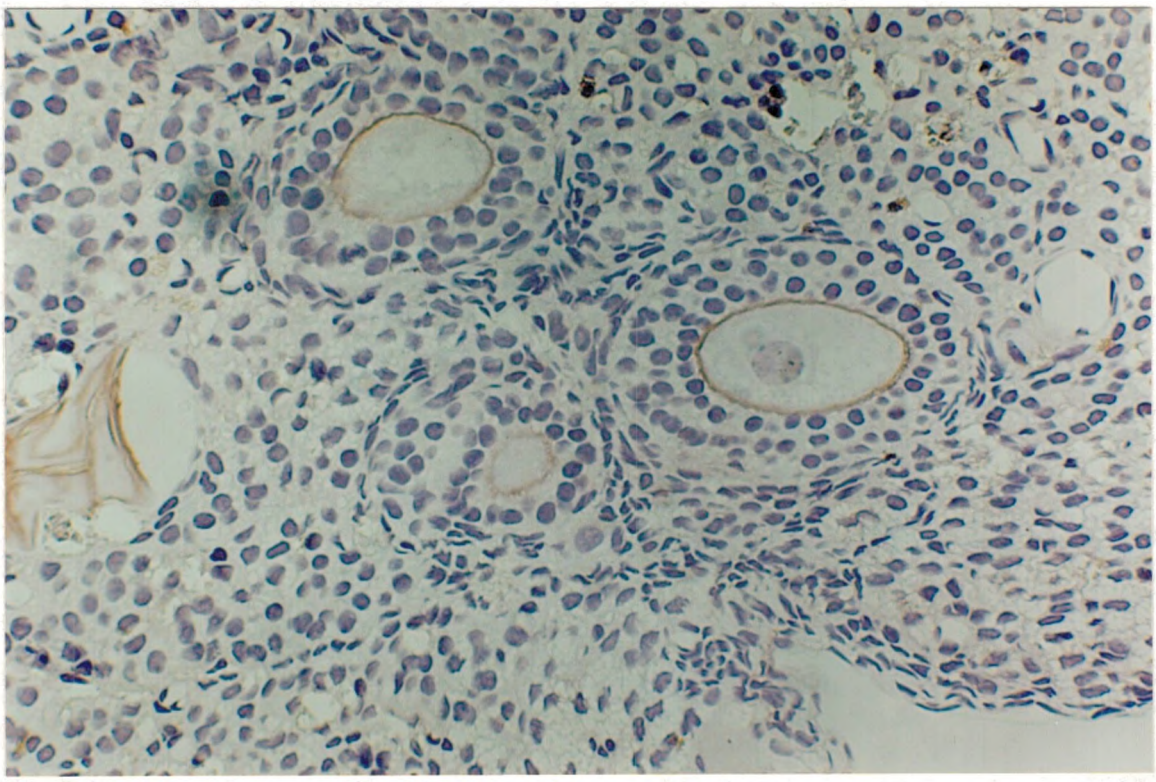
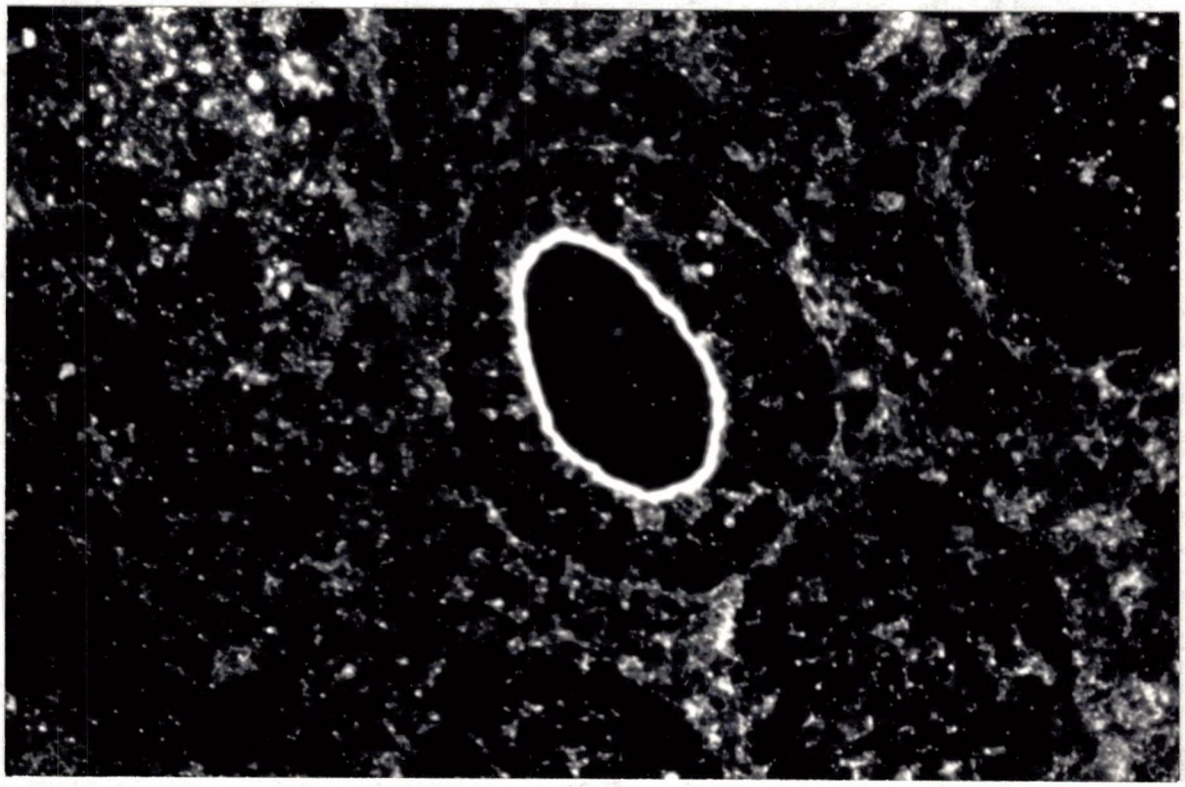


PLATE XII

Figure 23. Fluorescence micrograph of a follicle seen in an ovary harvested at 1400 hours on day four of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by FITC-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.

Figure 24. Fluorescence micrograph of follicles seen in an ovary harvested at 1400 hours on day four of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by FITC-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.



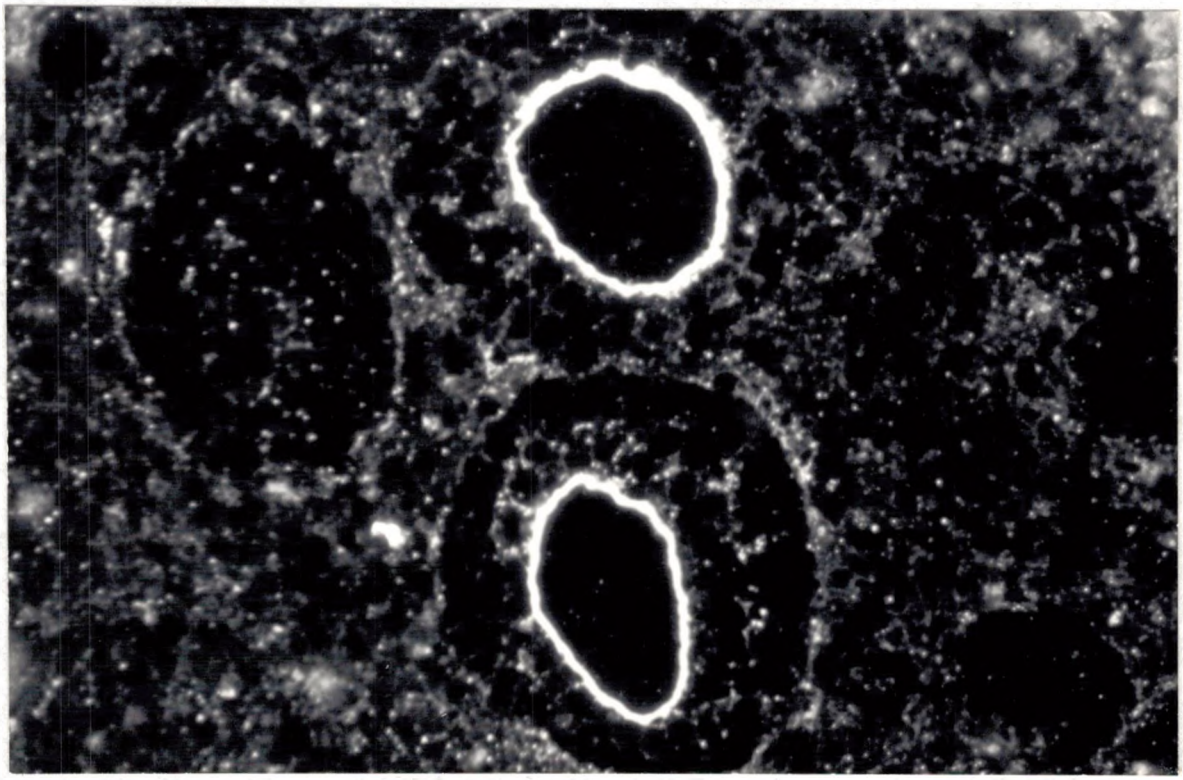
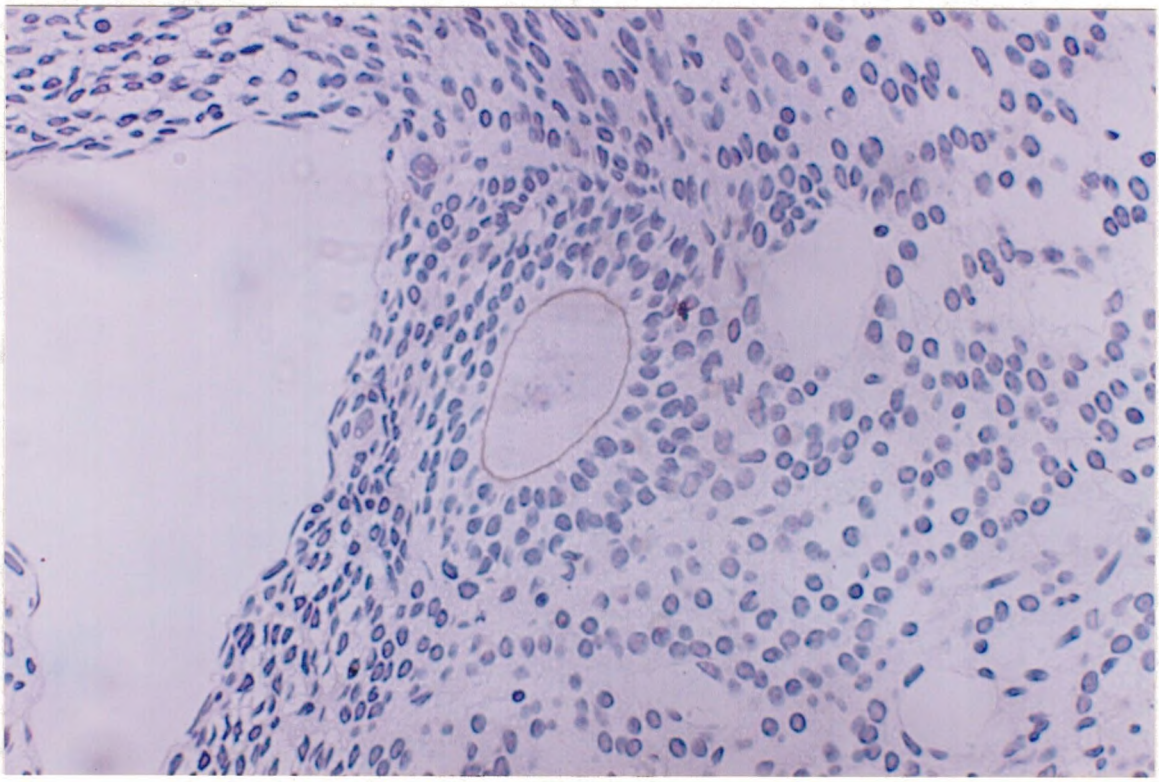


PLATE XIII

Figure 25. Light micrograph of a follicle seen in an ovary harvested at 2200 hours on day four of the estrous cycle. Treated with goat anti-mouse IgA as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 1800.

Figure 26. Light micrograph of a multilaminar follicle seen in an ovary harvested at 2200 hours on day four of the estrous cycle. Treated with goat anti-hamster IgG as a primary antibody followed by peroxidase-conjugated rabbit anti-goat IgG as a secondary antibody. X 4600.



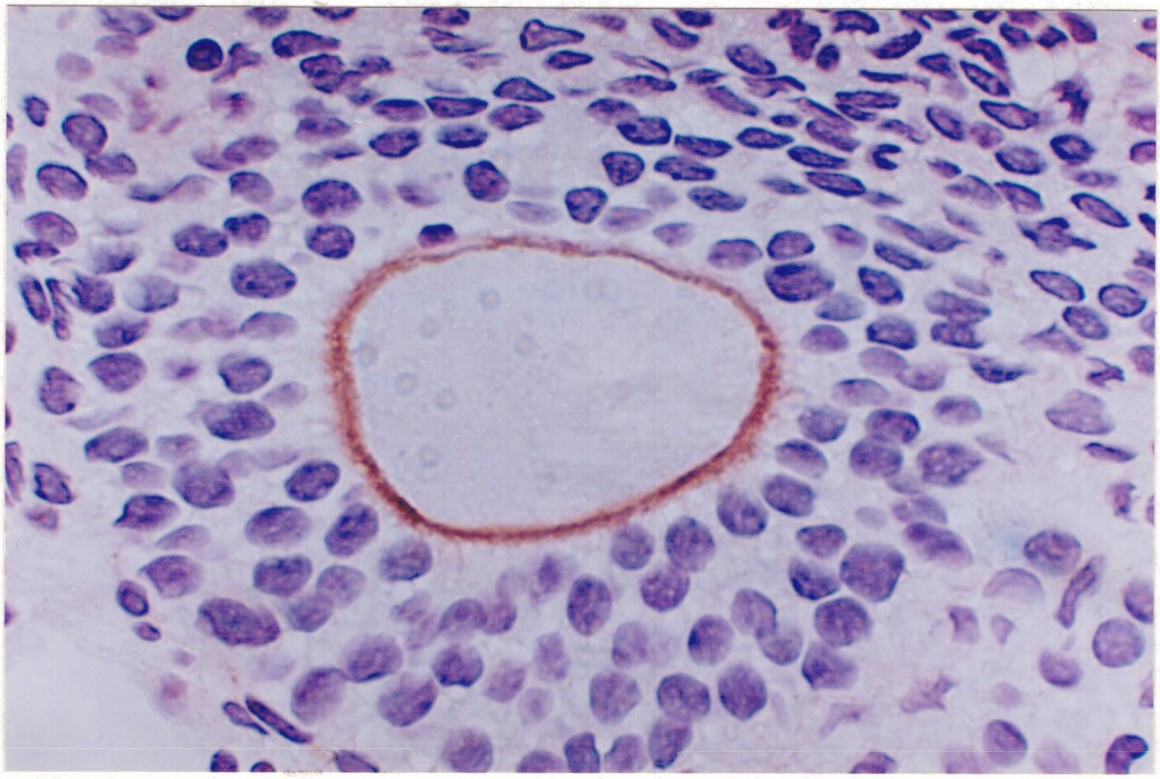
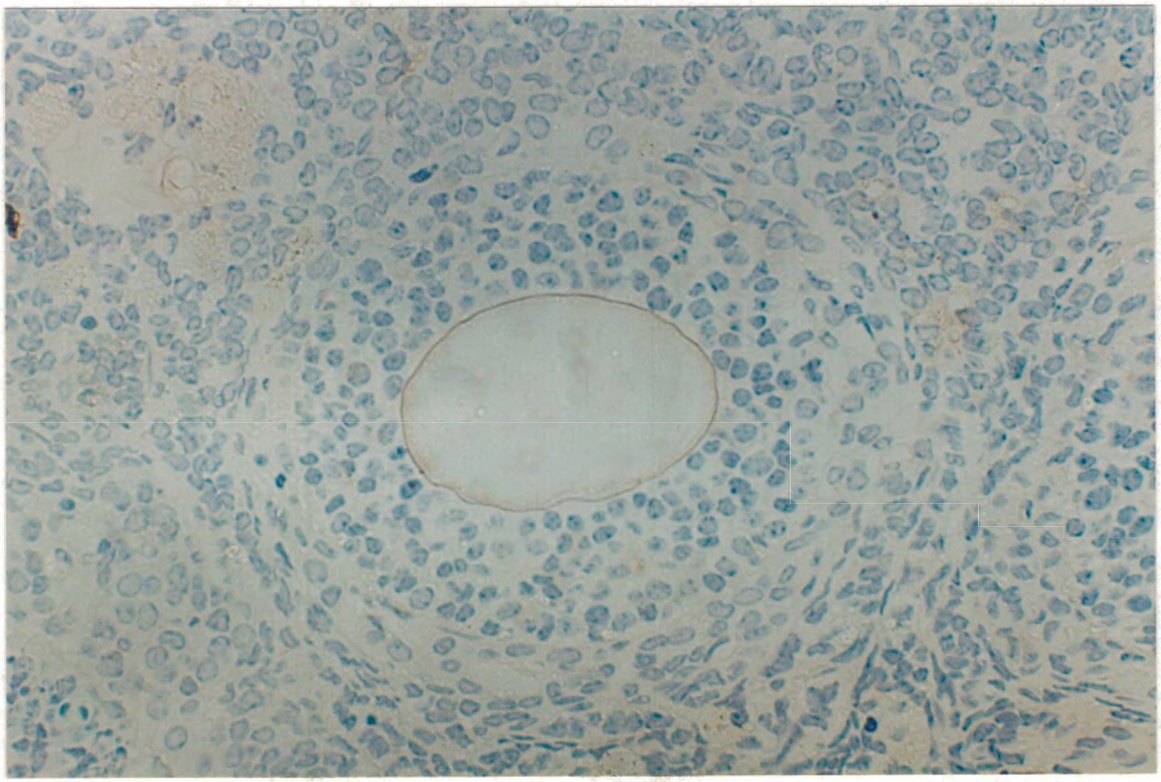


PLATE XIV

Figure 27. Light micrograph of a follicle seen in an ovary harvested at 1400 hours on day four of the estrous cycle. Treated with rabbit anti-FP as a primary antibody followed by peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody. X 1800.

Figure 28. Light micrograph of a follicle seen in an ovary harvested at 1400 hours on day three of the estrous cycle. Treated with rabbit anti-albumin as a primary antibody followed by peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody. X 1800.



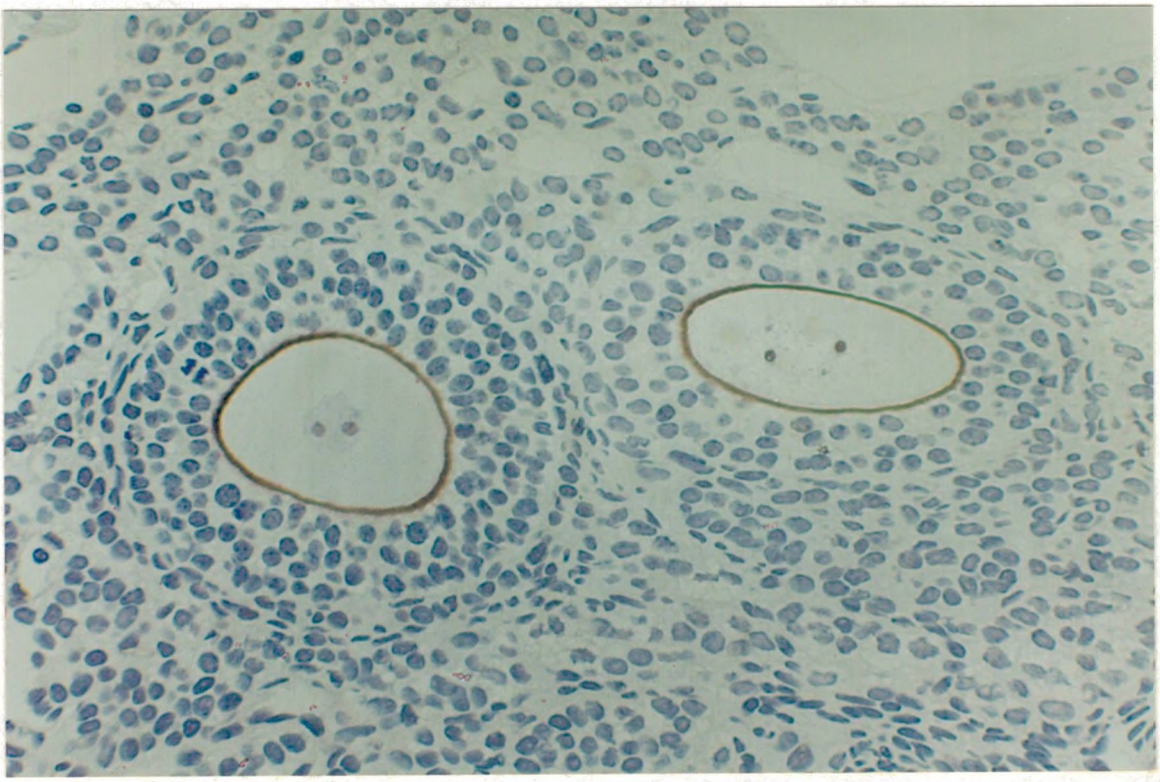
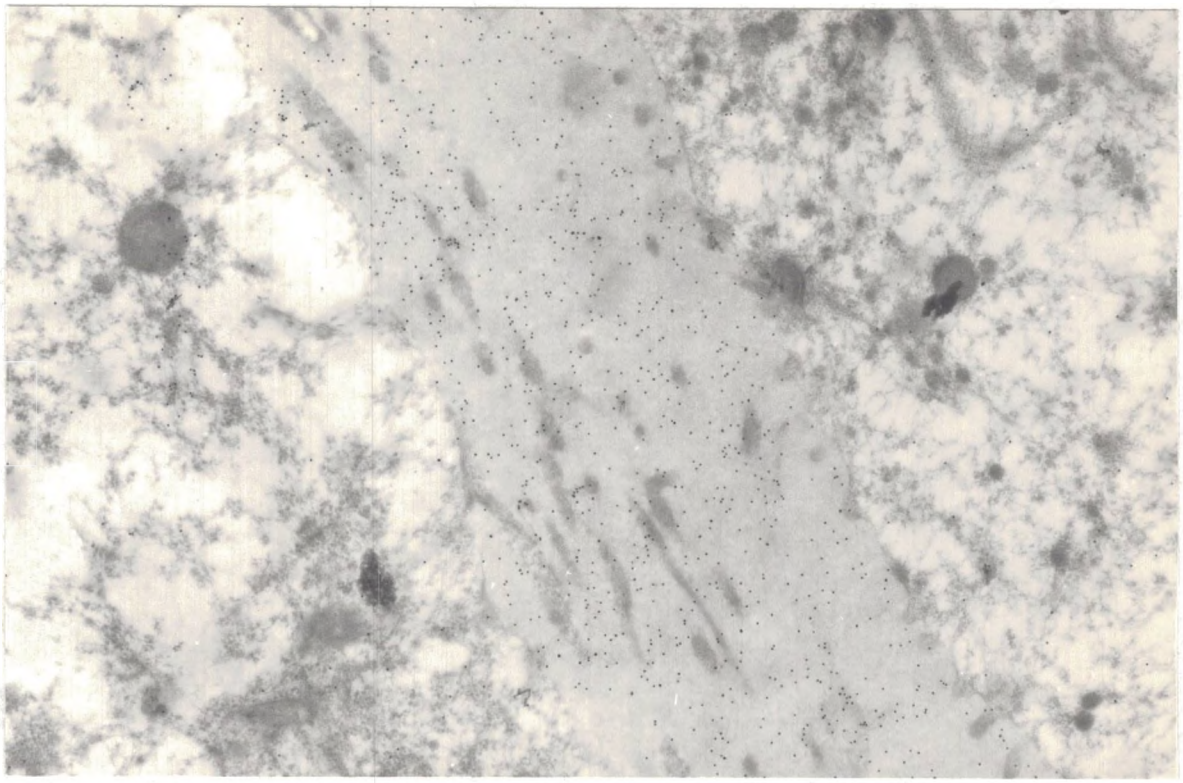


PLATE XV

Figure 29. Electron micrograph of the ZP of a follicle seen in an ovary harvested at 1400 hours on day four of the estrous cycle. Treated with rabbit anti-hamster albumin as a primary antibody followed by PAG-15. X 17,500.

Figure 30. Electron micrograph of the ZP of a follicle seen in an ovary harvested at 1400 hours on day four of the estrous cycle. Control tissue treated with PAG-15 only. X 16,100.



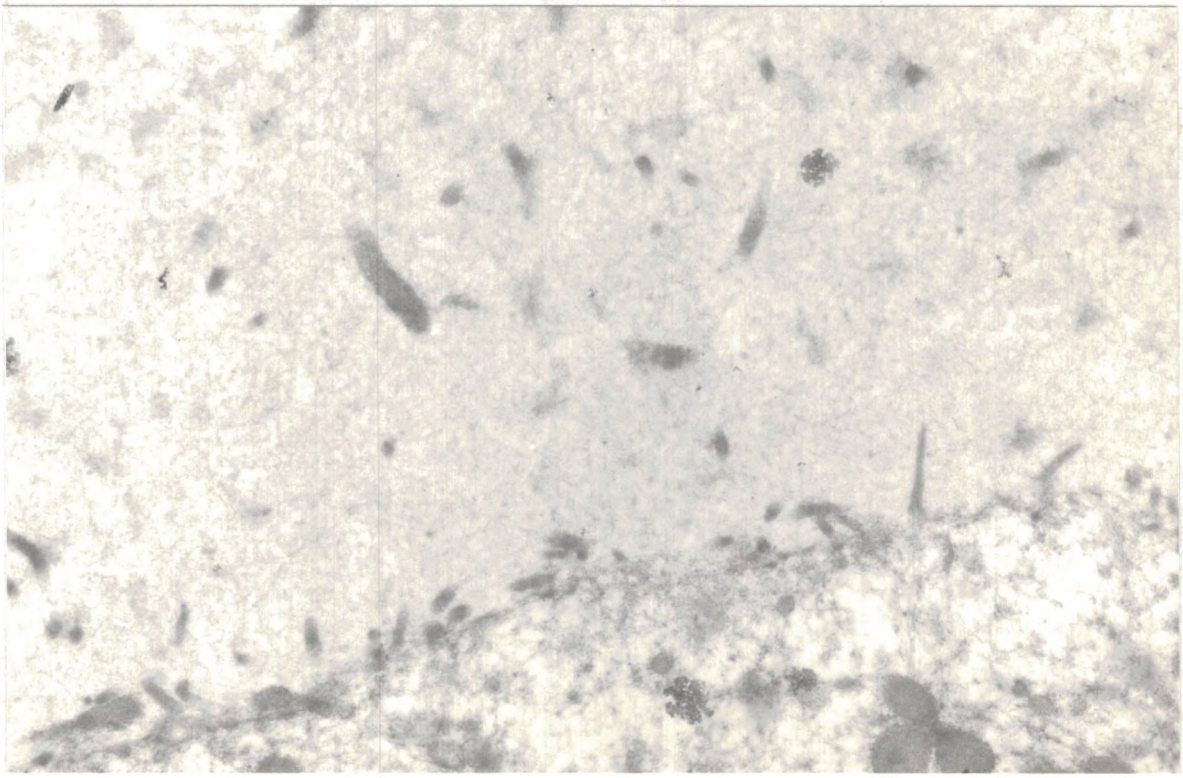
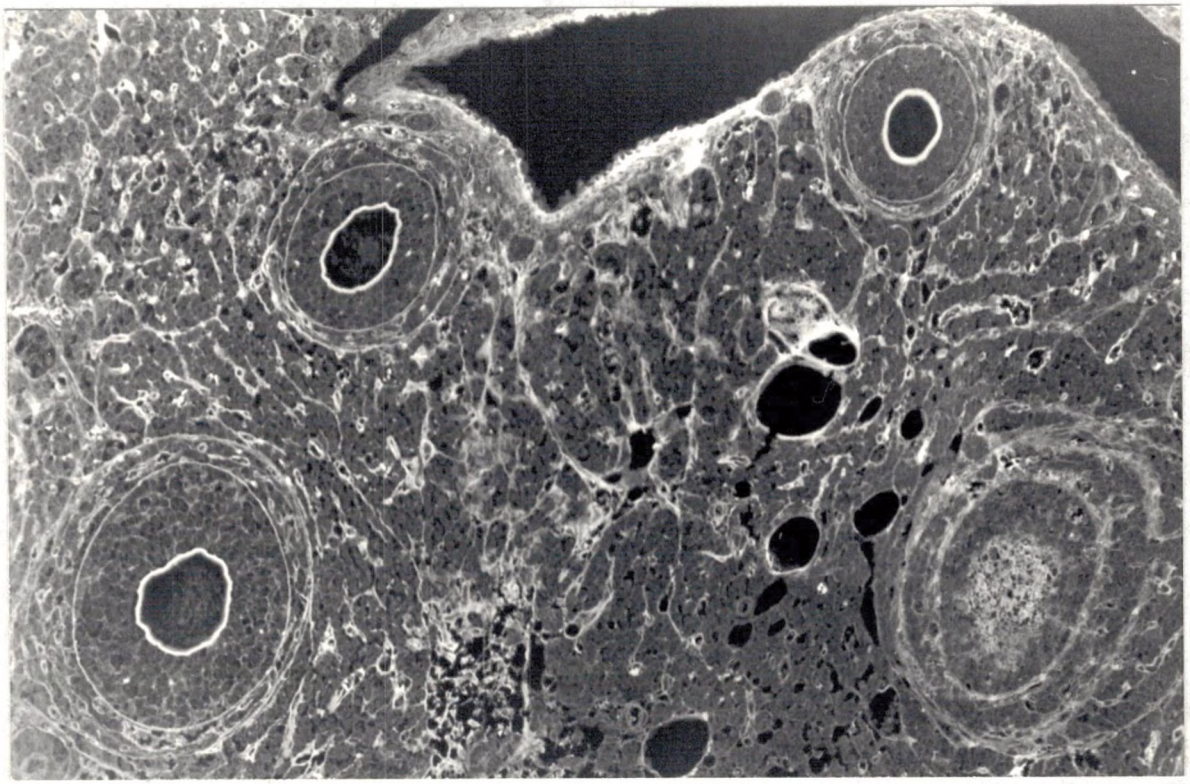


PLATE XVI

Figure 31. Fluorescence micrograph of a hamster ovary harvested at 1000 hours on day one of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note spots of fluorescence between granulosa cells denoting initial formation of liquor folliculi. X 460.

Figure 32. Fluorescence micrograph of a multilaminar follicle seen in an ovary harvested at 1000 hours on day one of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note the thin band of fluorescence at the level of the granulosa cell basement membrane. X 1800.



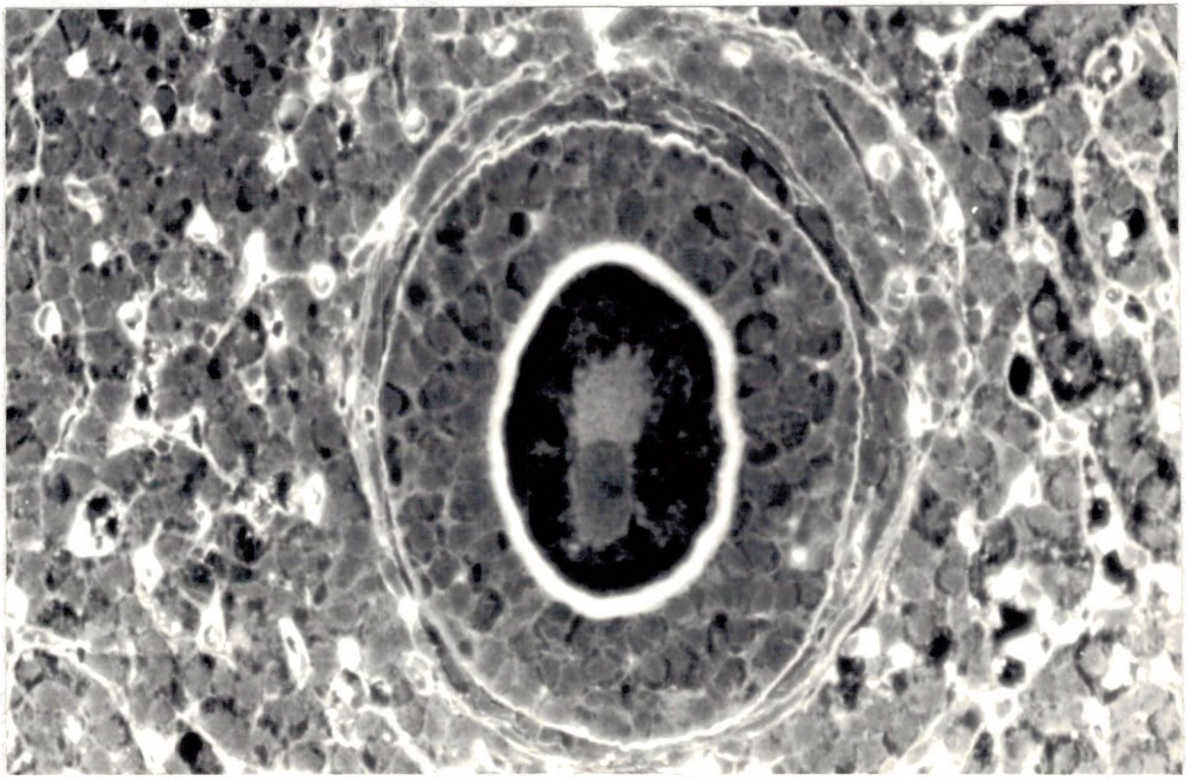
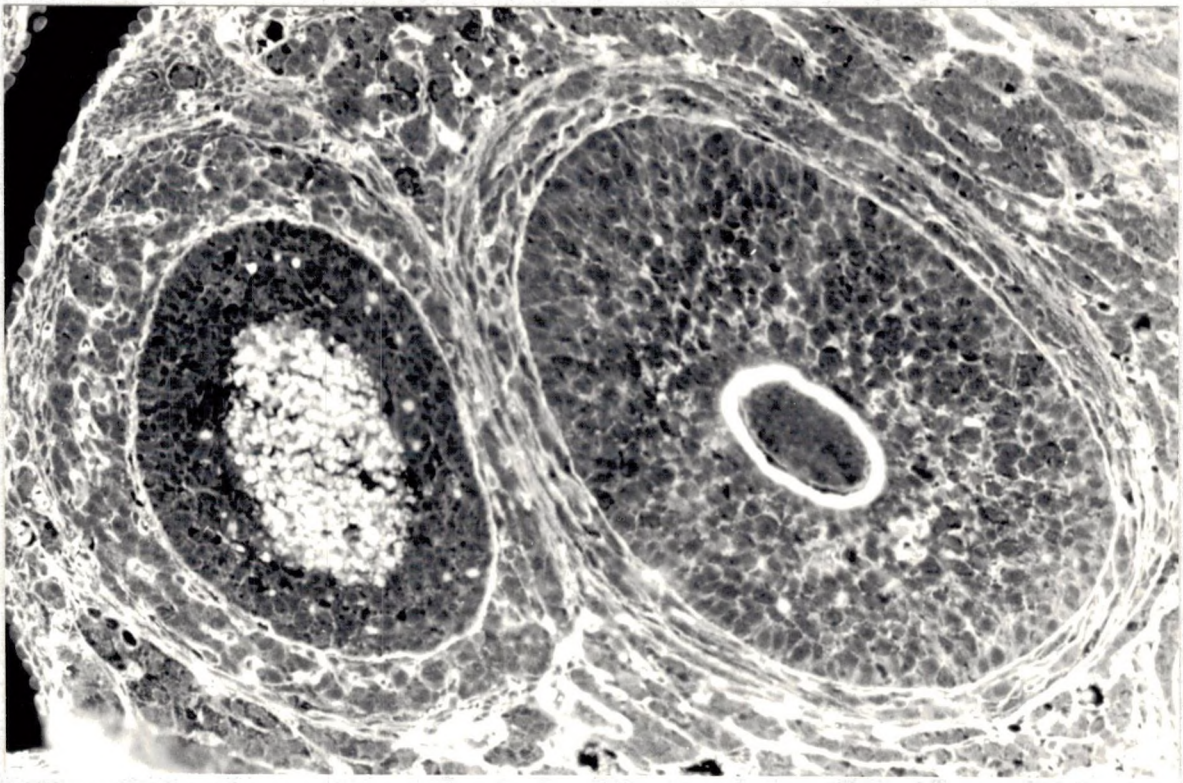


PLATE XVII

Figure 33. Fluorescence micrograph of two follicles seen in a hamster ovary harvested at 1000 hours on day one of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note the fluorescence between granulosa cells in the multilaminar follicle (right) and in the antrum of the antral follicle (left). X 900.

Figure 34. Fluorescence micrograph of an ovary harvested at 1000 hours on day one of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note the fluorescence seen in the antrum of the two antral follicles (center and left) as well as the lack of fluorescence in the remnants of zona pellucida atretica. X 460.



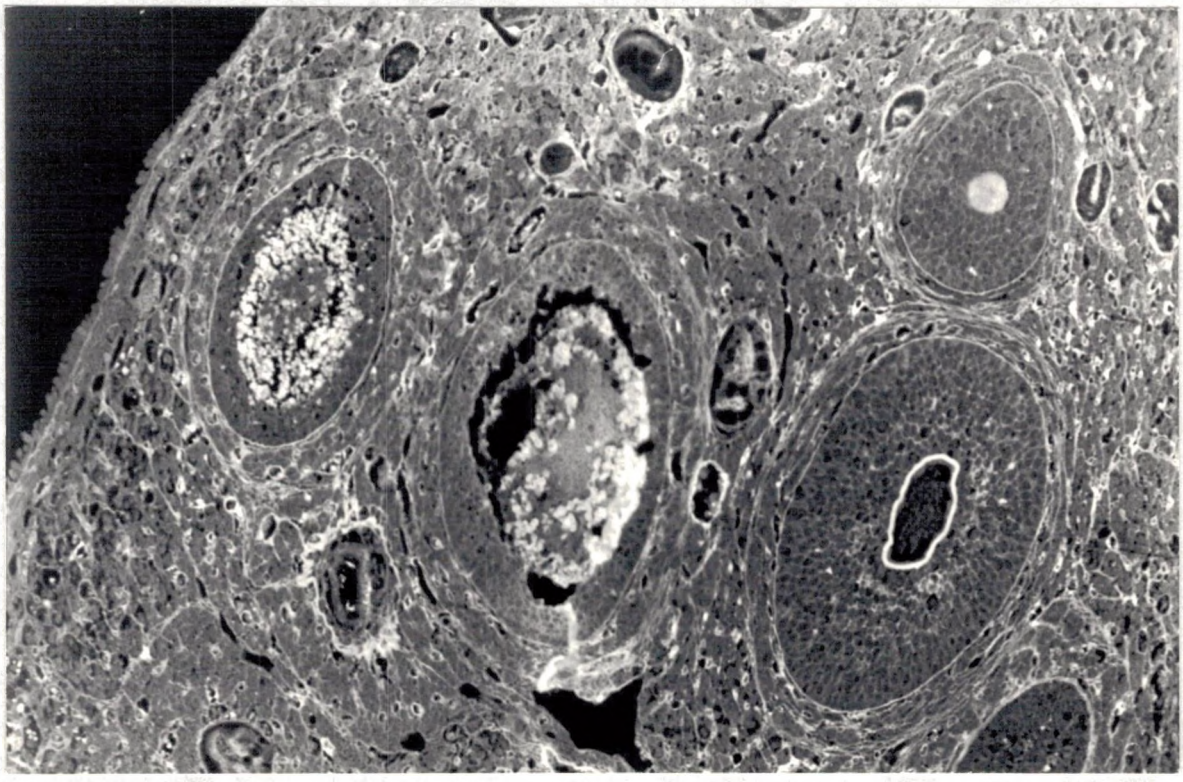
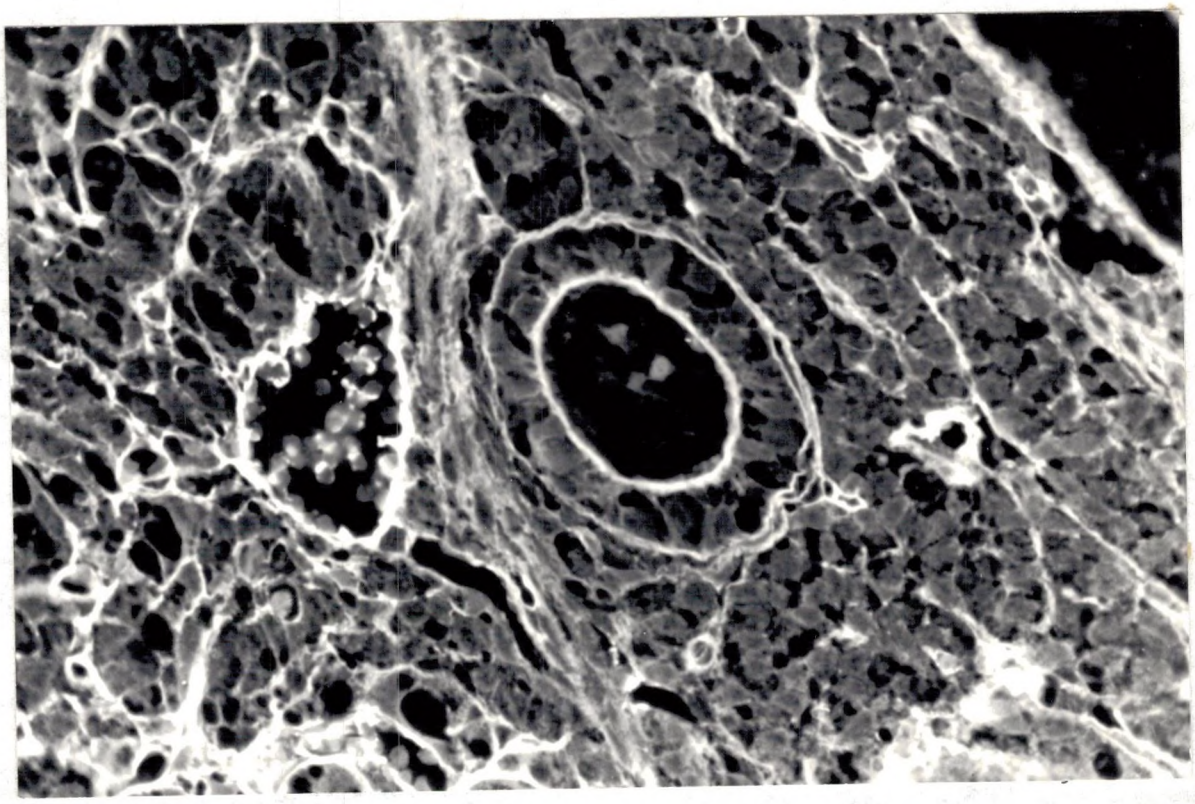


PLATE XVIII

Figure 35. Fluorescence micrograph of a bilaminar follicle seen in an ovary harvested at 1000 hours on day two of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. X 1800.

Figure 36. Fluorescence micrograph of a multilaminar follicle seen in an ovary harvested at 1000 hours on day two of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note areas of fluorescence between granulosa cells presumably marking initial formation of liquor folliculi. X 1800.



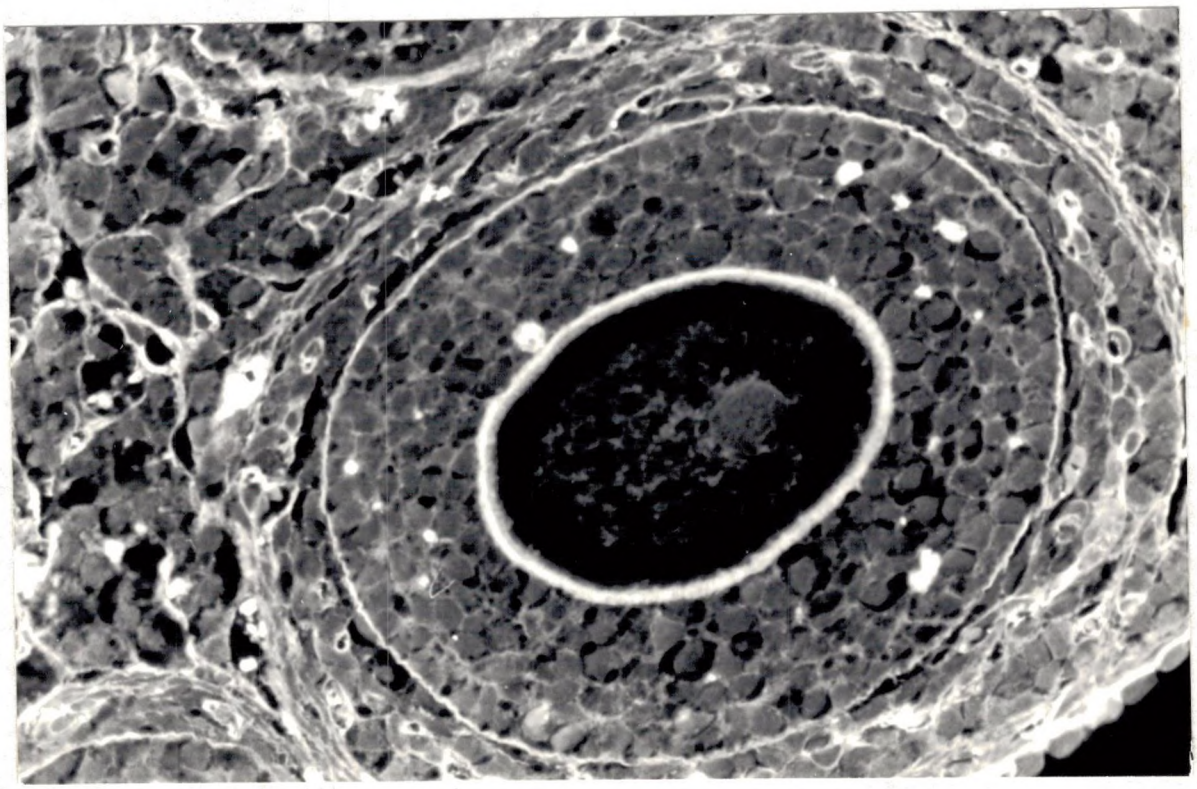
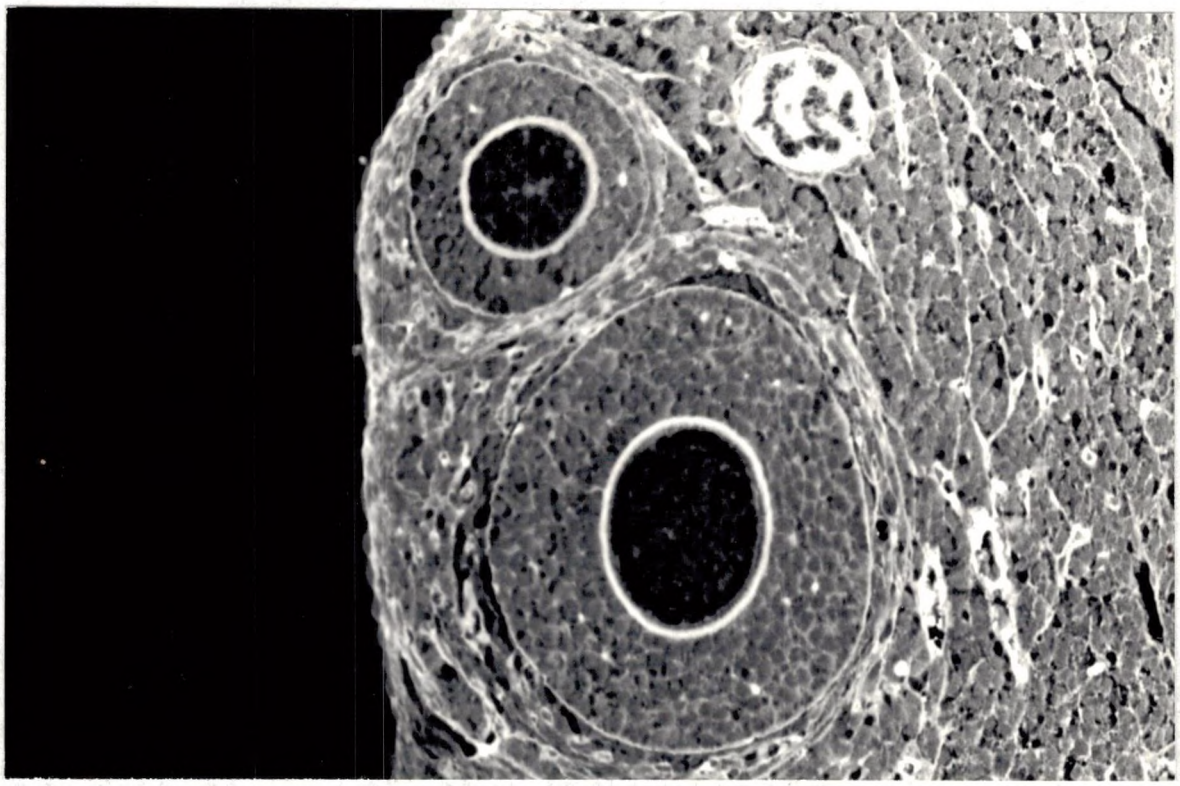


PLATE XIX

Figure 37. Fluorescence micrograph of two multilaminar follicles seen in an ovary harvested at 1000 hours on day two of the estrous cycle. The animal was injectd i.v. with FITC-conjugated hamster IgG one hour before euthanasia. X 900.

Figure 38. Fluorescence micrograph of a hamster ovary harvested at 1000 hours on day two of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. Note the lack of fluorescence of the remnants of zona pellucida atretica (center) as well as in the ZP of the atretic follicle (right). Also note spots of fluorescence on the oocyte coat in the atretic follicle. X 900.



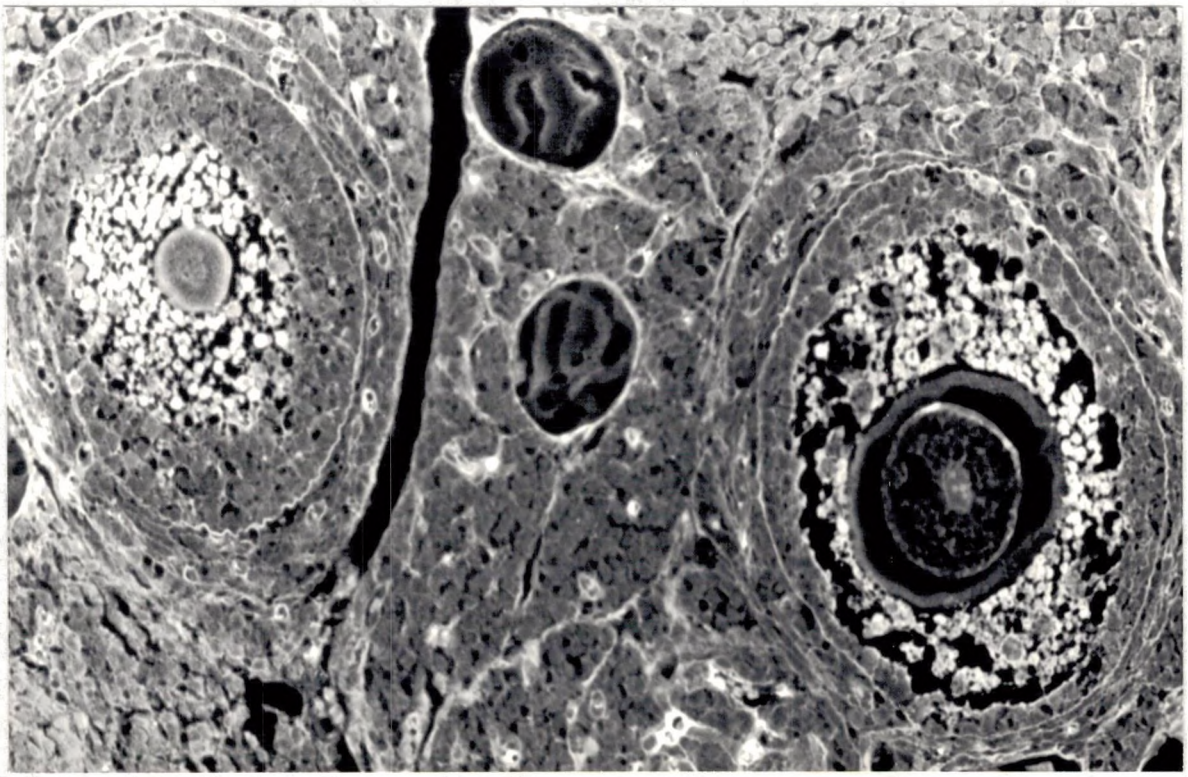
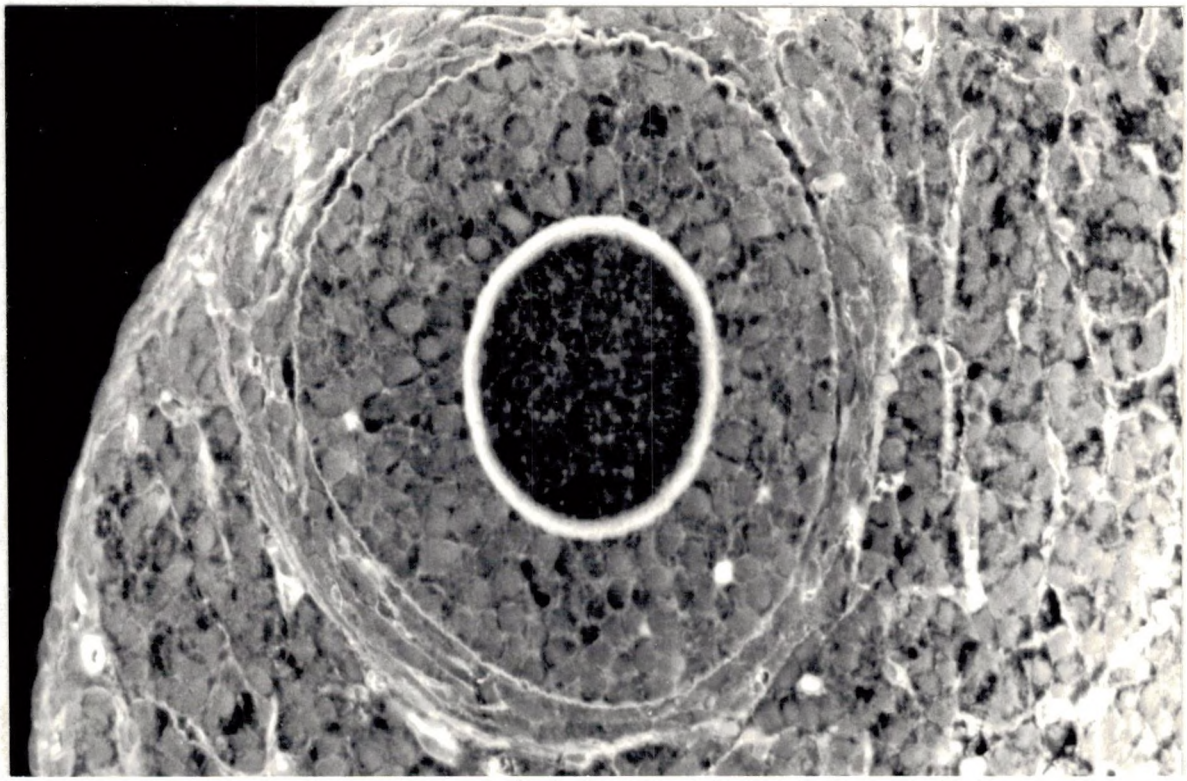


PLATE XX

Figure 39. Fluorescence micrograph of a multilaminar follicle seen in an ovary harvested at 1000 hours on day three of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG 30 minutes before euthanasia. X 1800.

Figure 40. Fluorescence micrograph of a multilaminar follicle seen in an ovary harvested at 1000 hours of day three of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG 30 minutes before euthanasia. X 1800.



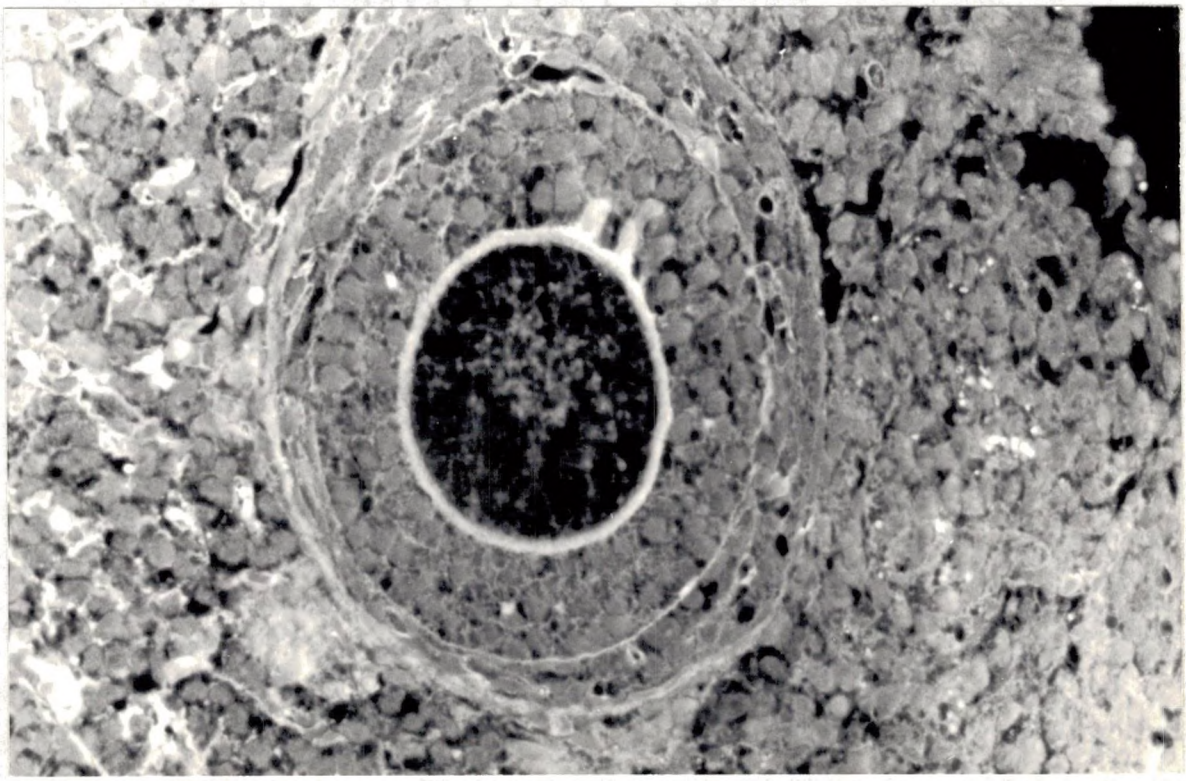
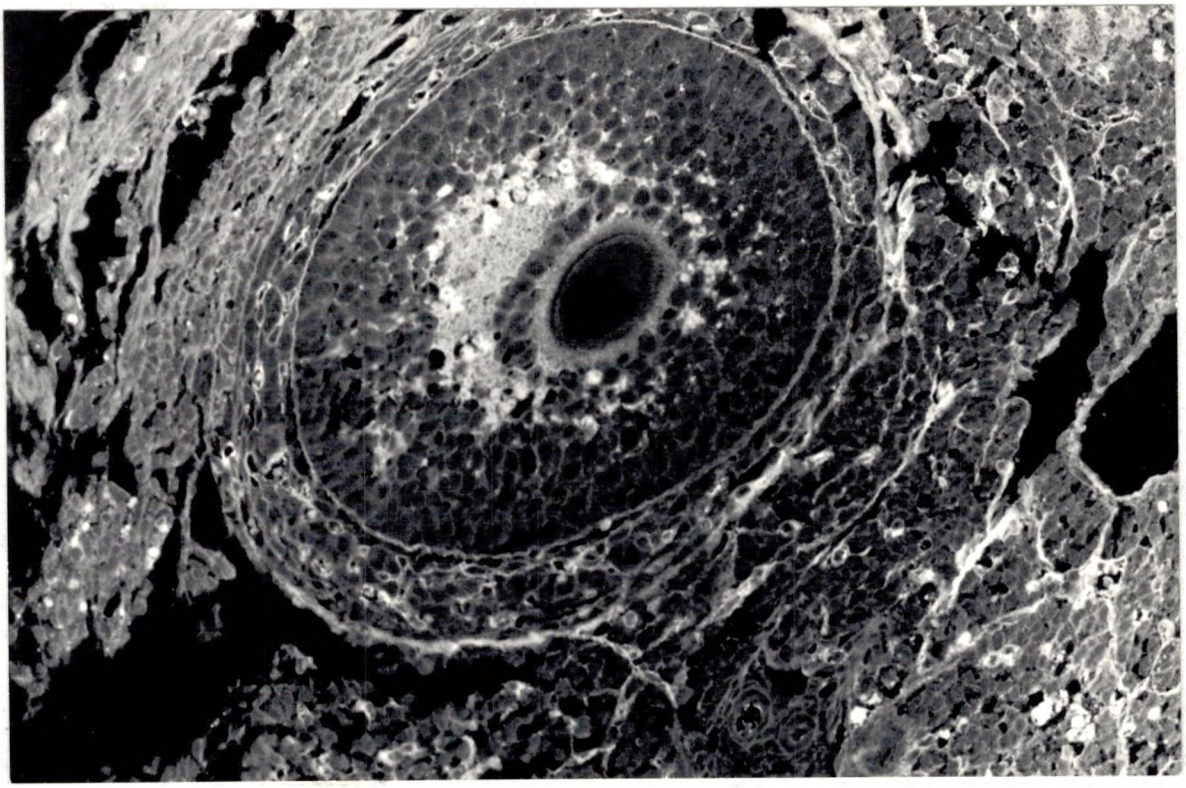


PLATE XXI

Figure 41. Fluorescence micrograph of an antral follicle as seen in an ovary harvested at 1000 hours on day three of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. X 900.

Figure 42. Fluorescence micrograph of an antral follicle seen in an ovary harvested at 1000 hours on day three of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. X 900.



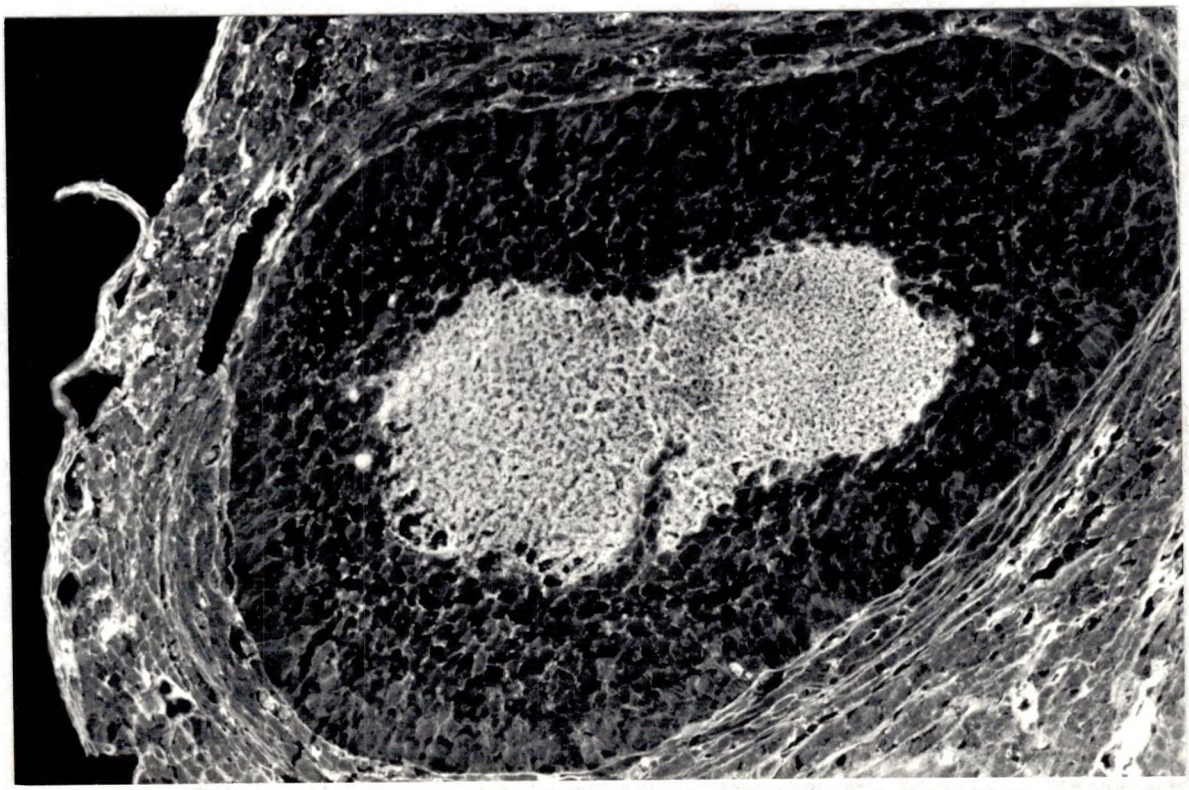
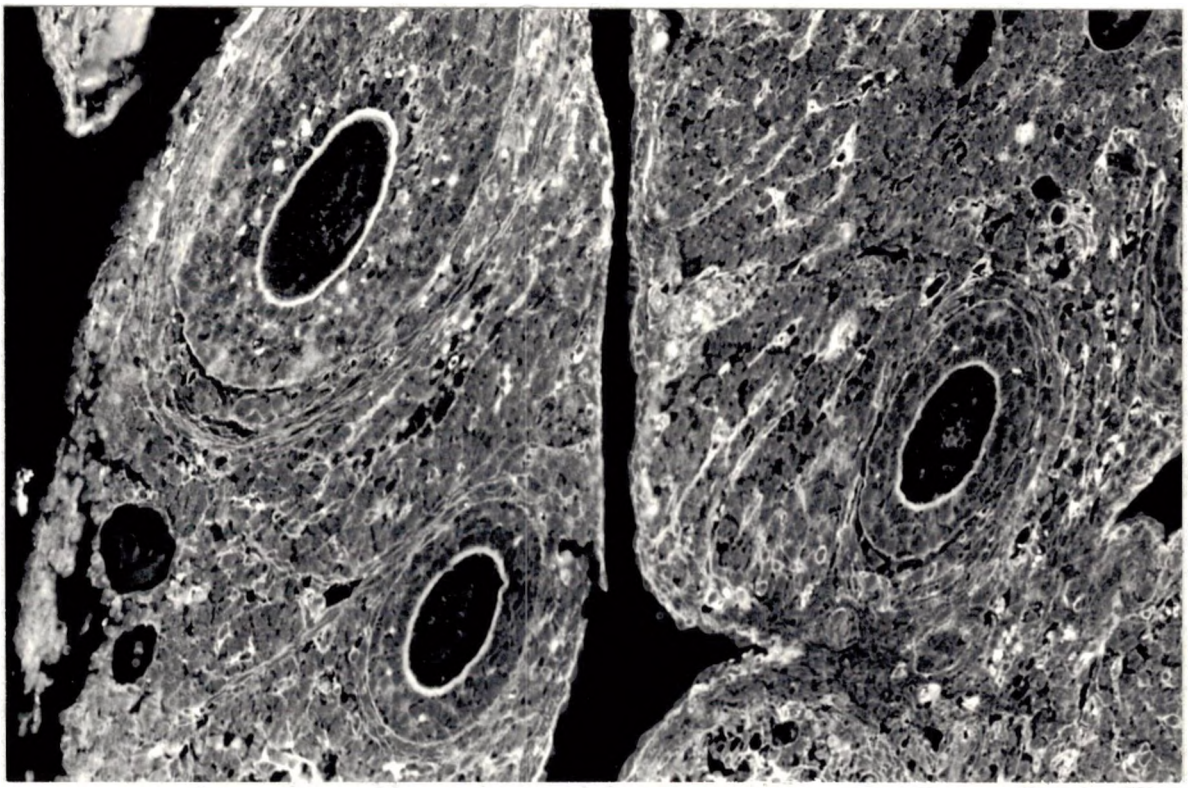
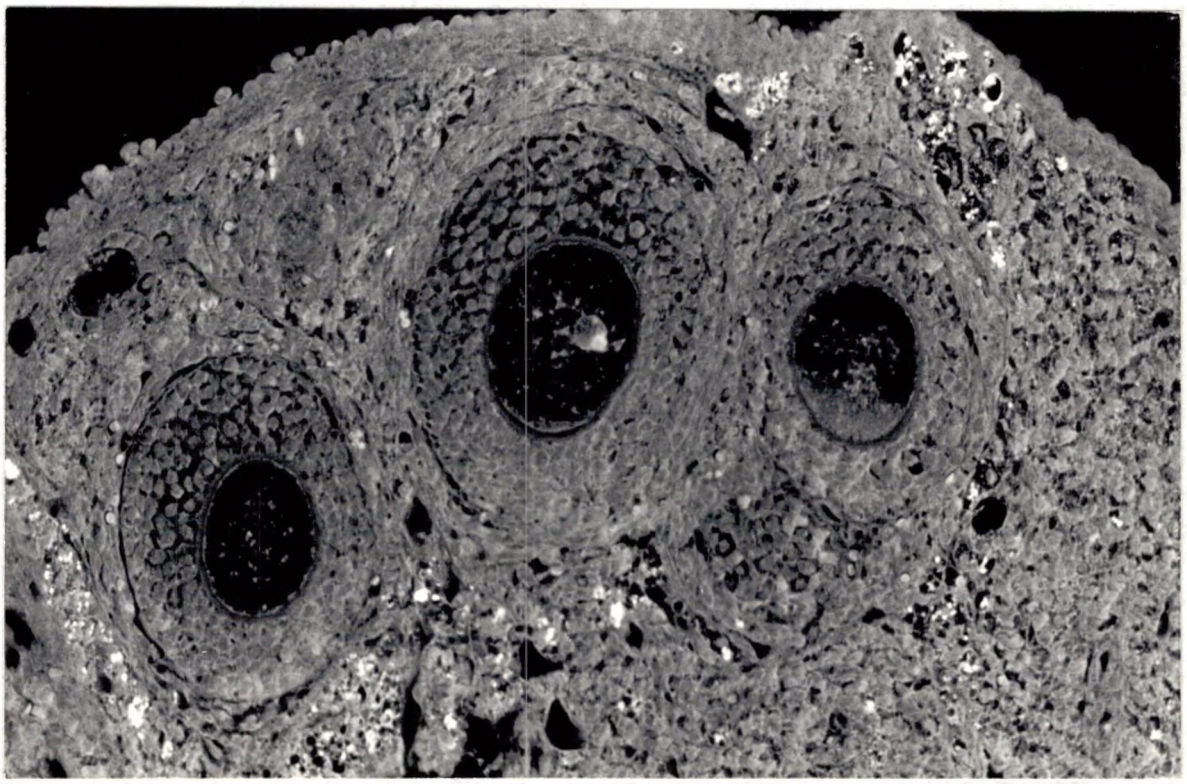


PLATE XXII

Figure 43. Fluorescence micrograph of three follicles seen in an ovary harvested at 1000 hours on day four of the estrous cycle. The animal was injected i.v. with FITC-conjugated hamster IgG one hour before euthanasia. X 900.

Figure 44. Fluorescence micrograph of three follicles seen in an ovary harvested at 1000 hours on day three of the estrous cycle. Control tissue - the animal was injected i.v. with FITC in physiologic saline one hour before euthanasia. X 900.





Appendix B Immunological Agents And Their Suppliers

Sigma Chemical Co. St Louis MO

Anti-Goat IgG (Whole Molecule) FITC Conjugate developed in rabbit

Anti-Rabbit IgG (Whole molecule) TRITC Conjugate developed in goat

Fluorescein Isothiocyanate

Cappel (Organon-Teknika) Durham NC

IgG fraction Goat Anti-Hamster IgG (Heavy and Light Chain Specific)

Goat Anti-Mouse IgA (Alpha Chain Specific)

Peroxidase Conjugated IgG fraction Rabbit Anti-Goat IgG (F(ab')2 Fragment Specific

Peroxidase Conjugated IgG fraction Goat Anti-Rabbit IgG (F(ab')2 fragment specific

Chromatographically purified Hamster IgG

Janssen (Amersham) Picataway NJ

Protein A Gold 15 nm

The following three antibodies were a generous gift from Dr. John Coe at the National Institute of Health, Rocky Mountain Laboratory, Hamilton, MT:

Rabbit Anti-Hamster IgG nonspecific

Rabbit Anti-Hamster Female Protein

Rabbit Anti-Hamster Albumin

LITERATURE CITED

Ahuja, K.K., and G.P. Bolwell 1983 Probable asymmetry in the organization of components of the hamster zona pellucida. Reprod. Fert., 69:49-55.

Altmann, L.G., B.G. Schneider, and D.S. Papermaster 1984 Rapid embedding of tissues in lowicryl K4M for immunoelectron microscopy. J. Histochem. Cytochem., 32:1217-1223.

Anderson, E. 1967 Observations on the uptake of horseradish peroxidase by developing oocytes of the rabbit. J. Cell Biol., 35:160A.

Anderson, E., and D.F. Albertini 1976 Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J. Cell Biol., 71:680-686.

Austin, C.R. 1961 The mammalian egg. Blackwell Scientific Publications, Oxford.

Austin, C.R., and J.E. Lovelock 1958 Permeability of rabbit, rat and hamster egg membranes. Exp. Cell Res., 15:260-261.

Beckstead, J.H. 1985 Optimal antigen localization in human tissues using aldehyde-fixed plastic-embedded sections. J. Histochem. Cytochem., 33:954-958.

Bevilacqua, M.P., J.S. Pober, M.E. Wheeler, D. Mendrick, R.S. Cotran, and M.A. Gimbrone 1985 Interleukin-1 (IL-1) acts on the vascular endothelial cells to increase their adhesivity for blood leukocytes. Fed. Proc., 44:1494 abst.

Bleil, J.D., and P.M. Wassarman 1980a Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev. Biol., 76:185-202.

Bleil, J.D., and P.M. Wassarman 1980b Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture in vitro. Proc. Natl. Acad. Sci. USA., 77:1029-1033.

Bleil, J.D., and P.M. Wassarman 1980c Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell, 20:873-882.

Bousquet, D., M.C. Leveille, K.D. Roberts, A. Chapdelaine, and G. Bleau 1981 The cellular origin of the zona pellucida antigen in the human and hamster. J. Exp. Zool., 215:215-218.

Brinster, R. 1965 Lactate dehydrogenase activity in the preimplanted mouse embryo. Biochim. Biophys. Acta., 110:439-44.

Britton, A.P., Y.S. Moon, and B.H. Yuen 1991 A simple handling technique for mammalian oocytes and embryos during preparation for transmission electron microscopy. J. Microscopy, 161:497-499.

Brower, P.T., and R.M. Schultz 1982 Biochemical studies of mammalian oogenesis: possible existence of a ribosomal and Poly(A)-containing RNA-protein supramolecular complex in mouse oocytes. J. Exp. Zool., 220:251-260.

Buckshee, K., and A. Mhaskar 1984 Detection of auto-antibodies to zona pellucida in human sera. Indian J. Med. Res., 80:288-292.

Chamberlin, M.E., M.J. Ringuette, C.C. Philpott, S.M. Chamow, and J. Dean 1989 Molecular genetics of the mouse zona pellucida. In: The mammalian egg coat structure and function. J. Dietl, ed. Springer-Verlag, New York, pp. 1-17.

Chiquoine, A.D. 1960 The development of the zona pellucida of the mammalian ovum. Am. J. Anat., 106:49-169.

Chiu, K.Y., and K.W. Chan 1987 Rapid immunofluorescence staining of human renal biopsy specimens using microwave irradiation. J. Clin. Path., 40:689-692.

Christofferson, R.H., and B.O. Nilsson 1988 Microvascular corrosion casting with analysis in the scanning electron microscope. Scanning, 10:43-63.

Clark, J.G. 1900 The origin, development and degeneration of the blood vessels of the human ovary. Johns Hopkins Hosp. Reports., 9:593-676.

Coe, J.E. 1977 A sex-limited serum protein of syrian hamsters: definition of female protein and regulation by testosterone. Proc. Natl. Acad. Sci. USA., 74:730-733.

Coe, J.E., S.S. Margossian, H.S. Slayter, and J.A. Sogn 1981 Hamster female protein a new pentraxin structurally and functionally similar to C-reactive protein and amyloid P component. J. Exp. Med., 153:977-991.

Coe, J.E., and M.J. Ross 1983 Hamster female protein a divergent acute phase protein in male and female syrian hamsters. J. Exp. Med., 157:1421-1433.

Coe, J.E., and M.J. Ross 1985 Hamster female protein, a sex-limited pentraxin, is a constituent of syrian hamster amyloid. J. Clin. Invest., 76:66-77.

Coe, J.E., and M.J. Ross 1990 Armenian hamster female protein: a pentraxin under complex regulation. Am. J. Physiol., 259:R341-R349.

Cooper, N.R. 1987 The complement system. In: Basic and clinical immunology (6th ed.). D.P. Stites, J.D. Stobo, and J.V. Wells eds., Appleton and Lange, Los Altos. pp.114-127.

Corvol, P., and C.W. Bardin 1973 Species distribution of testosterone binding globulin. Biol. Reprod., 8:277-284.

Croft, S.M., R.F. Mortensen, and H. Gewurz 1976 Binding of C-reactive protein to antigen-induced but not mitogen-induced T lymphoblasts. Science, 193:685-687.

Dietl, J. 1989 Ultrastructural aspects of the developing mammalian zona pellucida. In: The mammalian egg coat, structure and function. J. Dietl, ed. Springer-Verlag, New York, pp.49-60.

Dietl, J., and A.B. Czuppon 1984 Ultrastructural studies of the porcine zona pellucida during the solubilization process by Li-3.5-diiodosalicylate. Gamete Res., 9:45-54.

Doerr-Schott, J., and C. Lichte 1986 A triple ultrastructural immunogold staining method. J. Histochem. Cytochem., 34:1101-1104.

Drell, D.W., and B.S. Dunbar 1984 Monoclonal antibodies to rabbit and pig zonae pellucidae distinguish species-specific and shared antigenic determinants. Biol. Reprod., 30:445-457.

Dunbar, B.S. 1983a Morphological, biochemical, and immunochemical characterization of the mammalian zona pellucida. In: Mechanism and control of animal fertilization. J.F. Hartmann, ed. Academic Press, New York, pp. 139-175.

Dunbar, B.S. 1983b Antibodies to zona pellucida antigens and their role in fertility. In: Immunology of Reproduction. T.G. Wegman and T. J. Gil III, eds. Oxford University Press, London, pp. 505-534.

Dunbar, B.S., A.B. Dudkiewicz, and D.S. Bundman 1985 Proteolysis of specific porcine zona pellucida glycoproteins by boar acrosin. Biol. Reprod., 32:619-630.

Dunbar, B.S., C. Liu, and D.W. Sammons 1981 Identification of the three major proteins of porcine and rabbit zonae pellucidae by two-dimensional gel electrophoresis: comparison with follicular fluid, sera, and ovarian cell proteins. Biol. Reprod., 24:1111-1124.

Dunbar, B.S., G.A. Maresh, and K. Washenik 1989 Ovarian development and the formation of the mammalian zona pellucida. In: The mammalian egg coat, structure and function. J. Dietl, ed. Springer-Verlag, New York, pp. 38-60.

Dym, M. 1973 The fine structure of the monkey (Macaca) sertoli cell and its role in maintaining the blood-testis barrier. Anat. Rec., 175:639-656.

Dym, M. 1983 The male reproductive system. In: Histology Cell and Tissue Biology (5th ed.). L. Wiess, ed. Elsevier Press, New York, pp. 1000-1053.

Everett, N.B., and B. Simmons 1958 Measurement and radioautographic localization of albumin in rat tissues after intravenous administration. Circ. Res., 6:307-313.

Fiedel, B.A., and H. Gewurz 1976 Effects of C-reactive protein on platelet function. II. Inhibition by CRP of platelet reactivities stimulated by poly-L-lysine, ADP, epinephrine and collagen. J. Immunol., 117:1073-1078.

Ghitescu, L., A. Fixman, M. Simionescu, and N. Simionescu 1986 Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis. J. Cell Biol., 102:1304-1311.

Gilula, N.B., M.L. Epstein, W.H. Beers 1978 Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J. Cell Biol., 78:58-75.

Goodman, J.W. 1987 Immunoglobulins I: structure and function. In: Basic and clinical immunology (6th ed.). D.P. Stites, J.D. Stobo, and J.V. Wells eds., Appleton and Lange, Los Altos. pp. 27-36.

Greve, J.M., and P.M. Wassarman 1985 Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J. Mol. Biol., 181:253-264.

Guraya, S.S. 1974 Morphology, histochemistry, and biochemistry of human oogenesis and ovulation. Int. Rev. Cytol., 37:121-151.

Haddad, A., and M.E.T. Nagai 1977 Radioautographic study of glycoprotein biosynthesis and renewal in the ovarian follicle of mice and the origin of the zona pellucida. Cell Tissue Res., 177:347-369.

Hastings, R.A. II, A.C. Endeers, and S. Schlafke 1972 Permeability of the zona pellucida to protein tracers. Biol. Reprod., 7:288-296.

Heller, D.T., D.M. Cahill, R.M. Schultz 1981 Biochemical studies of mammalian oogenesis: metabolic cooperativity between granulosa cells and growing mouse oocyte. Dev. Biol., 84:455-464.

Hertig, A.T., and E.C. Adams 1967 Studies on the human oocyte and its follicle. J. Cell Biol., 34:647-675.

Heyner, S., R.L. Brinster, and J. Palm 1969 Effect of iso-antibody on preimplantation mouse embryos. Nature, 222:783-784.

Hokama, Y., M.K. Coleman, and R.F. Riley 1962 In vitro effects of C-reactive protein on phagocytosis. J. Bacteriol., 83:1017-1024.

Hope, J. 1965 The fine structure of developing follicles of the rhesus monkey. J. Ultrastuct. Res., 12:592-610.

Hornung, M., and S. Fritchi 1971 Isolation of C-reactive protein and its effect on human lymphocytes in vitro. Nature, 230:84-85.

Huang, S., H. Minassian, and J.D. More 1976 Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. Lab. Invest., 35:383-390.

Hussein, A.M., and F.J. Bourne 1984 Immunoglobulin concentrations in pig follicular fluid. Int. J. Fertil., 29:54.

Hutter, J.F., H.M. Piper, and P.G. Spieckermann 1984 Myocardial fatty acid oxidation: evidence of albumin-receptor-mediated membrane transfer of fatty acids. Basic Res. Cardiol., 79:274-282.

Kan, F.W.K., E. Roux, S. St.-Jacques, and G. Bleau 1990 Demonstration by lectin-gold cytochemistry of transfer of glycoconjugates of oviductal origin to the zona pellucida of oocytes after ovulation in hamsters. Anat. Rec., 226:37-47.

Kaplan, G., S. Abreu and R. Bachvarova 1982 rRNA accumulation and protein synthesis patterns in growing mouse oocytes. J. Exp. Zool., 220:361-370.

Kaplan, M.H., and J.E. Volanakis 1974 Interaction of C-reactive protein complexes with the complement system. J. Immunol., 112:2135-2147.

Kay, D.J., B. Boettcher, J.L. Yovich, and J.D. Stanger 1985 Antispermatozoal antibodies in human follicular fluid. Am. J. Reprod. Immunol. Microbiol., 7:113-117.

Kerjaschki, D., H. Sawada, and M.G. Farquahar 1986 Immunoelectron microscopy in kidney research: some contributions and limitations. Kidney Intl., 30:229-245.

Kindmark, C.O. 1971 Stimulating effect of C-reactive protein on phagocytosis of various species of pathogenic bacteria. Clin. Exp. Immunol., 8:941-948.

Kindmark, C.O. 1972 In vitro binding of C-reactive protein by some pathogenic bacteria and zymosan. Clin. Exp. Immunol., 11:283-289.

Krishna, A., and K. Spanel-Borowski 1989 Intracellular detection of albumin in the ovaries of golden hamsters by light and electron microscopy. Arch. Histol. Cytol., 52:387-393.

LaMarca, M.J., and P.M. Wassarman 1979 Ribosomal protein synthesis in mouse eggs and embryos. Dev. Biol., 73:103-119.

Larsson, L.-I. 1988 Immunocytochemistry: theory and practice. CRC Press, Boca Raton.

Leong, A.S.-Y., and J. Milios 1986 Rapid immunoperoxidase staining of lymphocyte antigens using microwave irradiation. J. Path., 148:183-187.

Leong, A.S.-Y., and J. Milios 1990 Accelerated immunohitochemical staining by microwaves. J. Path., 161:327-334.

Leveille, MC., K.D. Roberts, S. Chevalier, A. Chapdelaine, and G. Bleau 1987 Formation of the hamster zona pellucida in relation to ovarian differentiation and follicular growth. J. Reprod. Fert., 79:173-183.

Levo, Y., B. Frangione, and E.C. Franklin 1977 Amino acid sequence similarities between amyloid P component, C1t and CRP. Nature, 268:56-57.

Manarang-Pangan, S., and A.C. Menge 1971 Immunologic studies on human follicular fluid. Fert. Steril., 22:367-372.

Mancini, R.E., O. Vilar, J. Heinrich, O.W. Davidson, and B. Alvarez 1963 Transference of circulating labeled serum proteins to the follicle of the rat ovary. J. Histochem. Cytochem., 11:80-88.

Mangia, F., R.P. Erickson, and C. Epstein 1976 Synthesis of LDH-1 during mammalian oogenesis and early development. Dev. Biol., 54:146-150.

Maresh, G.A., and B.S. Dunbar 1987 Antigenic comparison of five species of mammalian zonae pellucidae. J. Exp. Zool., 244:299-307.

Matousek, J., J. Hojny, and A. Janik 1986 Serum allotypes in ovarian follicular fluids of pigs. Anim. Genet., 17:169.

Millette, C.F., and A.R. Bellve 1977 Temporal expression of membrane antigens during mouse spermatogenesis. J. Cell Biol., 74:86-97.

Mori, T., T. Nishimoto, H. Kohda, I. Takai, T. Nishimura, and T. Oikawa 1979 A method for specific detection of autoantibodies to the zona pellucida in infertile women. Fertil. Steril., 32:67-72.

Moore, K.L. 1988 The developing human; clinically oriented embryology. (4th ed.). W.B. Saunders, Philadelphia.

Oakberg, E.F., and P.D. Tyrell 1975 Labeling the zona pellucida of the mouse oocyte. Biol. Reprod., 12:477-482.

Ockner, R.K., R.A. Weisiger, and J.L. Gollan 1983 Hepatic uptake of albumin-bound substances: albumin receptor concept. Am. J. Physiol., 245:G13-G19.

Oikawa, T. 1989 Analysis of the antigenicity of hamster zona pellucida with a monoclonal antibody. In: Reproductive immunology 1989: Proceedings of the 4th international congress. L. Mettler, and W.D. Billington eds., Elsevier Science, New York, pp. 109-116.

Osmand, A.P., B. Friedenson, H. Gewurz, R.H. Painter, T. Hofmann, and E. Shelton 1977 Characterization of C-reactive protein and the complement subcomponent C1t as homologous proteins displaying cyclic pentameric symmetry (pentraxins). Proc. Natl. Acad. Sci. USA., 74:739-743.

O'Rand, M.G., and L.J. Romrell 1977 Appearance of cell surface auto- and isoantigens during spermatogenesis in the rabbit. Dev. Biol., 55:347-358.

Pepys, M.B., M. Baltz, K. Gomer, A.J.S. Davies, and M. Doenhoff 1979 Serum amyloid P-component is an acute phase reactant in the mouse. Nature, 278:259-261.

Pepys, M.B., A.C. Dash, T.C. Fletcher, N. Richardson, E.A. Munn, and A. Feinstein 1978 Analogues in other mammals and in fish of human plasma proteins, C-reactive protein and amyloid P component. Nature, 273:168-170.

Perricone, R., C. De Carolis, C. Moretti, E. Santuari, G. De Sanctis, and L. Fontana 1990 Complement, complement activation and anaphylatoxins in human ovarian follicular fluid. Clin. Exp. Immunol., 82:359-362.

Phillips, D.M., and R.M. Shalgi 1980 Surface properties of the zona pellucida. J. Exp. Zool., 213:1-8.

Philpott, C.C., M.J. Ringuette, and J. Dean 1987 Oocyte specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. Dev. Biol., 121:568-575.

Pino, R.M., and C.L. Thouron 1983 Vascular permeability in the rat eye to endogenous albumin and immunoglobulin G (IgG) examined by immunohistochemical methods. J. Histochem. Cytochem., 31:411-416.

Pollanen, P.P., and B.P. Setchell 1989 Microvascular permeability in the rat testis at puberty. Int. J. Androl., 12:206-218.

Rodewald, R., and J.-P. Kraehenbuhl 1984 Receptor mediated transport of IgG. J. Cell Biol., 99:159s-164s.

Rothschild, M.A., M. Oratz, and S.S. Schreiber 1972 Albumin Synthesis. New England J. Med., :748-757, 816-821.

Sacco, A.G. 1979 Inhibition of fertility in mice by passive immunization with antibodies to isolated zonae pellucidae. J. Reprod. Fertil., 56:533.

Sacco, A.G. 1981 Immunocontraception: consideration of the zona pellucida as a target antigen. In: Obstetrics and gynecology annual, vol 10. R.M. Wynn, ed., Appleton-Century-Crofts, New York, p 1.

Sacco, A.G., M.G. Subramanian, E.C. Yurewicz, F.J. DeMayo, and W.R. Dukelow 1983 Heteroimmunization of squirrel monkeys (Saimiri sciureus) with a purified porcine antigen (PPZA): immune response and biologic activity of antiserum. Fertil. Steril., 39:350.

Sacco, A.G., E.C. Yurewicz, M.G. Subramanian, and F.J. DeMayo 1981 Zona pellucida composition: species cross reactivity and contraceptive potetial of antiserum to a purified pig zona antigen (PPZA). Biol. Reprod., 25:997-1008.

Sadler, T.W. 1985 Langman's medical embryology (5th ed.). Williams and Wilkins, Baltimore.

Sage, H., C. Johanson, and P. Bornstein 1984 Characterization of a novel serum albumin-binding glycoprotein secreted by endothelial cells in culture. J. Biol. Chem., 259:3993-4007.

Sakai, Y., Y. Araki, T. Yamashita, S. Kurata, T. Oikawa, M. Hiroi, and F. Sendo 1988 Inhibition of in vitro fertilization by a monoclonal antibody reacting with the zona pellucida of the oviductal egg but not the ovarian egg of the golden hamster. J. Reprod. Immunol., 14:177-189.

Schnitzer, J.E., W.W. Carley, and G.E. Palade 1988 Specific albumin binding to microvascular endothelium in culture. Amer. J. Physiol., 254:H425-437.

Schultz, R.M., G.E. Letourneau, and P.M. Wassarman 1979 Program of early development in the mammal: Changes in the patterns and absolute rates of tubulin and total protein synthesis during oocyte growth in the mouse. Dev. Biol., 73:120-133.

Sellens, M.H., and E.J. Jenkinson 1975 Permeability of the mouse zona pellucida to immunoglobulins. J. Reprod. Fert., 42:153-157.

Shalgi, R., P. Kraicer, A. Rimon, M. Pinto, and N. Soferman 1973 Proteins of the human follicular fluid: the blood-follicle barrier. Fert. Steril., 24:429-434.

Sharpe, R.M. 1979 Gonadotropin-induced accumulation of "interstitial fluid" in the rat testis. J. Reprod. Fert., 55:365-373.

Shimizu, S., M. Tsuji, and J. Dean 1983 In vitro biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicular culture. J. Biol. Chem., 258:5858-5863.

Shivers, C.A., and B.S. Dunbar 1977 Autoantibodies to zona pellucida: a possible cause for infertility in women. Science. 197:1187-1190.

Shteri, P.K., J.T. Murai, G.L. Hammond, J.A. Nisker, W.J. Raymoure, and R.W. Kulin 1982 The serum transport of steroid hormones. Rec. Progr. Horm. Res., 38:457-510.

Siegel, J., R. Rent, and H. Gewurz 1974 Interactions of C-reactive protein with the complement system. Protamine-induced consumption of complement in acute phase sera. J. Exp. Med., 140:631-647.

Simionescu, N. 1981 Transcytosis and traffic of membranes in the endothelial cell. In International Cell Biology 1980-1981. H.G. Schweiger, ed., Springer-Verlag, Berlin. 657-672.

Simmons, R.L., and P.S. Russell 1966 The histocompatibility antigens of fertilized mouse eggs and trophoblast. Ann. N.Y. Acad. Sci., 129:35-45.

Skinner, S.M., T. Mills, H.J. Kirchick, and B.S. Dunbar 1984 Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. Endocrinology, 115:2418-2432.

Sternberger, L.A. 1986 Immunocytochemistry (3rd ed.). John Wiley and Sons, New York.

Steward, M.W. 1974 Outline studies in biology: Immunochemistry. Goodwin, T.W. and J.M. Ashworth, eds. Halsted Press, New York.

Syed, V., O. Soder, S. Arver, M. Lindh, S. Khan, and M. Ritzen 1988 Ontogeny and cellular origin of an interleukin-1-like factor in the reproductive tract of the male rat. Int. J. Androl., 11:437-447.

Szego, M.C. 1975 Lysosomal function in nucleoplasmic communication. In Lysosomes in Biology and Pathology, Vol. 4. Dingle, J.T. and R.T. Dean, eds., Elsevier Publishers, New York. 385-477.

Tesoriero, J.V. 1977 Formation of the Chorion (zona pellucida) in the teleost, orysias latipes. II. Polysaccharide cytochemistry of early oogenesis. J. Histochem. Cytochem., 25:1376-1380.

Timmons, T.M., G.A. Maresh, D.S. Bundman, and B.S. Dunbar 1987 Use of specific monoclonal and polyclonal antibodies to define distinct antigens of the porcine zonae pellucidae. Biol. Reprod., 36:1275-1287.

Van de Winkel, J.G.J., and C.L. Anderson 1991 Biology of human immunoglobulin G Fc receptors. J. Leukocyte Biol., 49:511-524.

Wassarman, P.M. 1987a Early events in mammalian fertilization. Annu. Rev. Cell Biol., 3:109-142.

Wassarman, P.M. 1987b The biology and chemistry of fertilization. Science, 235:553-560.

Wassarman, P.M., and W.J. Josefowicz 1978 Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and miotic competence. J. Morphol., 156:209-236.

Williams, S.K., J.J. Devemy, and M.W. Bittensky 1981 Micropinocytotic ingestion of glycosylated albumin by isolated microvessels: possible role in pathogenesis of diabetic microangiopathy. Proc. Natl. Acad. Sci. USA., 78:2393-2397.

Williams, S.K., and D.A. Greener 1984 Adsorbtive endocytosis of glycosylated serum albumin by capillary endothelium. J. Cell Biol., 99 suppl:371a (Abstr).

Wolgemuth, D., J. Celenza, D. Bundman, and B.S. Dunbar 1984 Formation of the rabbit zona pellucida and its relationship to ovarian follicular development. Dev. Biol., 106:1-14.

Wood, H.F. 1951 Effect of C-reactive protein on normal human leukocytes. Proc. Soc. Exp. Biol. Med., 76:843-847.

Wood, H.F. 1953 Relationship between the acute phase response and antibody production in the rabbit. I. Correlation between the early appearance of Cx-reactive protein and subsequent antibody production. J. Exp. Med., 98:311-319.

Yokota, S. 1983 Immunocytochemical evidence for transendothelial transport of albumin and fibrinogen in rat heart and diaphragm. Biomed. Res., 4:577-586.

Yurewicz, E.C., A.G. Sacco, and M.G. Subramanian 1987 Structural characterization of the Mr=55,000 antigen (ZP3) of porcine oocyte zona pellucida. J. Biol. Chem., 262:564-571.

Zamboni, L. 1971 Fine morphology of mammalian fertilization. Harper and Row, New York.