Ultrastructural Study on Protein Permeability Into the Ovarian Follicles of the Mouse

Andrew F. Payer
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ULTRASTRUCTURAL STUDY ON PROTEIN PERMEABILITY INTO
THE OVARIAN FOLLICLES OF THE MOUSE

by
Andrew F. Payer

A Dissertation Submitted to the Faculty of the
Graduate School of Loyola University
in Partial Fulfillment of the
Requirements for the
Degree of
Doctor of Philosophy

February, 1974

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BIOGRAPHY

Andrew F. Payer was born in Pittsburgh, Pennsylvania, on August 20, 1943. In 1960, he graduated from Swissvale High School in Swissvale, Pennsylvania; and continued his studies at Edinboro State College, Edinboro, Pennsylvania. He received a Bachelor of Science degree in Biology in June, 1965, from Edinboro State College.

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Andrew F. Payer was married to Elizabeth Ann Trench on August 21, 1969.

During the writing of his dissertation he accepted
a teaching/research position in the Department of Anatomy at the University of Texas Medical Branch at Galveston, Texas.
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ABSTRACT

The purpose of this endeavor was to observe the permeability characteristics of the follicular apparatus in mice ovaries to systemically injected protein tracers. Particular emphasis was placed on the study of the perifollicular capillary bed.

The experimental animal was the female albino mouse. Some were in the prepubertal phase while others were sexually mature in the proestrous and metestrous phases of the estrous cycle. The macromolecular tracers utilized were ferritin and horseradish peroxidase (HRP), both suspended in normal saline solution and injected into the tail vein.

The capillaries of the perifollicular region were found to possess a continuous endothelium and continuous basement membrane, irrespective of the follicle size in immature mice, or the stage of the estrous cycle in the sexually mature animals.

Light microscopic studies confirmed the passage of the tracers from the capillaries into the follicle. In addition it confirmed the lack of gross ferritin leakage from the microcirculation due to altered permeability.
Electron microscopic observations enabled the precise localization of the penetration of both tracers from the capillary bed into the follicles.

HRP was seen to leave the capillaries primarily through the interendothelial cell junctions, and was present in all follicles within 30 seconds following administration of the tracer. Cellular uptake was observed in both the granulosa cells and the oocyte.

Ferritin, on the other hand was absent from the endothelial cell junctions. It left the capillaries at a slower rate than HRP, and apparently through cytoplasmic vesicular transport. The only other sites that appeared to slow ferritin passage, as opposed to HRP, were the tight intercellular spaces between the granulosa cells of unilaminar follicles. While follicles were observed to be permeable to the tracer, oocyte uptake was more prevalent in unilaminar follicles and was rarely found in the oocytes of more mature follicles.

Measurements taken on the percentage of ferritin in the pericapillary region demonstrate a proportional elevation in amount with respect to increased circulation times for the tracer.

These findings demonstrate: (i) that there is the
difference in the manner of exit of the two tracers from the systemic circulation into the follicles, along with their incorporation into the granulosa cells and into the oocyte; (ii) that the follicular apparatus is permeable to macromolecular tracers which are in the size range of serum proteins; (iii) that the morphology of the perifollicular capillaries does not change with respect to the stage of follicular development or reproductive state of the animal; (iv) that the basal lamina surrounding the mouse ovarian follicle is permeable to ferritin.
I. INTRODUCTION

This dissertation is a study on the morphology of follicular permeability in the mouse ovary with reference to systemically injected protein tracers, with the purpose of establishing whether permeability differences exist in the ovarian follicles of the immature, proestrus, and metestrus mouse.

While conducting earlier experiments on the mammalian ovary at the ultrastructural level and becoming familiar with the literature in this area, it became increasingly evident that little information has accumulated on the ultrastructural level concerning the microvasculature of this organ and the permeability of its follicular apparatus to protein molecules.

There have been numerous studies regarding hormone interactions of the ovary with other organs, studies on the mechanism of ovulation, investigations into the question of follicular atresia, and also ovarian pathology. But, besides the possible speculations by investigators, very little work has been conducted relating the above with the microvasculature of the ovary. It is possible that investigators may have assumed the capillary bed of
the ovary to be similar in function and structure to the microvasculature of other organs. But it is becoming more evident with each succeeding study that this is not necessarily so. Not only is there a variation between the capillary beds of various organs, but there is also accumulating evidence which indicates variations in the behavior of vessels under different physiological or pathological conditions (Florey, 1968). The investigation by Fawcett et al. (1970) on the structural components of the blood-testis barrier stimulated the idea of utilizing electron microscopy along with tracer techniques to study the permeability characteristics of the microvasculature in the mammalian ovary. The study by Fawcett revealed for the first time, base line information about the vascular bed of the interstitial tissue in the testis and the possible barriers to systemically injected particulate tracers that may be found there.

Therefore this thesis was concerned with: (1) an account of the ultrastructural morphology of the follicular apparatus; (2) the dynamics of the vasculature in the perifollicular region; (3) the use of tracers to study the permeability of this system; (4) the relationships between the extremes of the estrous cycle and any changes in the ovarian microvasculature that may correspond to it;
(5) ultimately to establish the pattern of tracer penetration and cellular uptake in the follicles, which can be correlated to different tracer circulation times in the animals.

The maturing ovarian follicle can be regarded as an extravascular structure, when referring to the area enclosed by the basal lamina. This statement is valid irrespective of follicular size or the state of follicular maturation. The significance of this morphological arrangement is that every molecule destined to enter the follicle has to leave the vascular bed of the perifollicular region, penetrate the thecal layer (if present), and then the basal lamina in order to become available for possible incorporation by the granulosa cells. To reach the oocyte, molecules may be transported by the granulosa cells or passively advance through the intercellular spaces and antrum. In the more mature follicles the oocyte is surrounded by a zona pellucida, which also may act as a regulator of molecular permeability to the oocyte. The ultimate question is whether the oocyte itself incorporates the molecules.

Investigators have been studying the variety of molecular types and sizes that penetrate through the basal lamina of the follicle in order to determine the metabolic
needs of the follicular cells and the possible harmful effects the penetrating molecules may induce. The goal of these early studies was to establish whether macromolecules could enter the ovarian follicle and to observe the extent of their uptake into the follicles and the cells they contain. Such studies were conducted at the light microscopic level.

Light microscopy produces a general view of protein penetration but its results are not comparable to the detailed observation possible with the electron microscope because the resolution of the instrument sets a limit of 250 millimicrons. The advantage of using tracers at the ultrastructural level is that they enable the student to visually observe the extent and manner of the protein penetration.

With regard to ultrastructural studies on macromolecular transport into the follicle the papers of Morris and Sass (1966), Anderson (E. 1967, 1972), and Anderson (W. 1972), should be cited and that their observations are compared to the results of this study.

Among the macromolecules used colloidal tracers were rejected because of their non-biological nature and the wide range of particle size; e.g. the range for carbon is 200-500 Å (Biozzi et al., 1953), the size for
colloidal gold is 30-250 A (Palade, 1961), and mercuric sulfide is 70-350 A (Majno and Palade, 1961).

The macromolecules chosen for these present studies were horseradish peroxidase (HRP) and ferritin. The HRP was chosen because it is a well established tracer of a known molecular weight (approx. 40,000, Keiline and Hartree, 1951) and molecular size (approx. 25-30 A, Karnovsky, 1967). Ferritin also has an established use in many investigations. It has a molecular weight of approximately 500,000, and a molecular size of approximately 110 A (Farrant, 1954; Harrison, 1959).
II. LITERATURE REVIEW

The literature review has been subdivided in order to present the material more effectively. The four subdivisions considered are: (A) the morphological changes of the ovary; (B) the morphology and function of the capillary; (C) the use of tracers in permeability studies and (D) the effects of hormones on the vasculature.

These articles were chosen for review in order to provide the background for studies conducted by citing the most important papers on the ovary and to illustrate the paucity of information on the permeability of the ovarian follicle to macromolecules. In addition, there will be a description of new tracer methods in electron microscopy which can be used in this paper to acquire a better understanding about the permeability of the follicular apparatus.

A. Morphological Changes in the Ovary

This segment can be further subdivided into: (1) morphology; (2) dynamics of the ovarian follicle; (3) permeability of the follicular apparatus; and (4) vasculature of the ovary.
1. The Morphology of the Ovary

The classical descriptions on the histology of the mouse ovary along with its cyclic changes were first published by Allen (1922) and Brambell (1928). Allen correlated ovarian changes regarding follicular growth and ovulation with the different stages of the estrous cycle. Brambell described the histology of the developing and mature mouse ovarian follicle. Since that time, the ovarian histological features have become well established and are reviewed by Harrison (1962). Examples of more recent investigations dealing with the ultrastructure of the developing follicles of the mammalian ovary are: Weakley (1966; 1967) on the hamster ovary; Hadek (1963), Odor and Blandau (1969), Peters (1969), and Byskov (1969) on the mouse ovary; Blanchette (1961) on the rabbit ovary; Bjorkman (1962), Franchi and Mandl (1962), and Flerko et al. (1967) on the rat; Anderson and Beams (1960) on the guinea pig; Hertig and Adams (1967) on the human ovary; and Hadek (1965) and Norrevang (1968) in review.

Since this paper deals with mouse ovary, studies conducted on the mouse will be reviewed at a somewhat greater length. The following papers to be presented describe the morphological characteristics of the mouse ovarian follicle from fetus life to maturity. Odor and Blandau
(1969) described the ultrastructure of the follicles of the early postnatal mouse ovary. They presented the histogenesis and cytogenesis of the oogonia along with the subsequent envelopment of the oogonia by the follicle cells. Peters (1969) then described the development of the mouse ovary from birth to maturity. This included the classification of the oocytes (size) and follicles (number of granulosa cells). The paper by Byskov (1969) was concerned with the mouse follicle in the "expansion" stage just prior to ovulation. The paper included a description of the ultrastructural appearance and changes occurring in the perifollicular region of this type of follicle. It also showed that the follicle (inside of the basal lamina) is avascular up to the time of corpus luteum formation, while the theca interna and externa are well vascularized.

2. Dynamics of the Ovarian Follicle

The classical papers on follicular development (Brambell, 1928) and follicular changes during the estrous cycle of the mouse (Allen, 1922) and the rat (Long and Evans, 1922; Boling et al., 1941) were descriptive studies illustrating the morphological characteristics of the follicles at different stages of development. However, the more recent literature is concerned with the
dynamics of the follicle such as: follicular growth rates, volume changes in follicles, and the intrinsic and extrinsic factors affecting development and maturation of the oocyte. It is this literature that will now be reviewed.

Pederson and Peters (1968) presented a system for the classification of follicles according to the number of granulosa cells found in the largest cross section of each follicle. This study was the foundation which led to the later works on the dynamics of follicle growth, including the time it takes a follicle to grow from one stage of development to another. Pederson (1970a, 1970b) used the above method in order to determine the follicular kinetics in the immature and mature cycling mouse. From the results of that study it became evident that ovarian follicles can be divided into two groups: the proliferating follicle pool and the non-proliferating follicle pool. The two pools can be correlated with the estrous cycle of the mouse in order to estimate the follicle types that would be found at a given stage of the estrous cycle (Pedersen, 1970a, 1970b).

A factor which should now be introduced and is associated with the morphological changes occurring in the ovarian follicle, is the serum hormone levels in the animal. It was shown by use of radioimmunoassay techniques that
there are changes in the serum LH and FSH levels in the rat (Daane and Parlow, 1971) and in the mouse (Kovacic and Parlow, 1972) that correspond to a given phase of the sexual cycle. Bingel and Schwartz (1969) demonstrated some variation in pituitary LH content and LH release which was correlated with variations in the reproductive organs of the mouse during the estrous cycle. In addition, there is evidence that variations in the serum levels of FSH and LH caused by extrinsic factors will produce a morphological effect on the ovaries; e.g. Jones and Nalbandov (1972) found that intrafollicular injections of gonadotrophins will alter follicular morphology; Goldman and Mahesh (1968, 1969), using antibodies to FSH and LH, induced changes in follicular morphology and incidence of ovulation.

This thesis is not a specific study of the above hormone interactions, but is an attempt to correlate possible changes taking place in the microcirculation of the follicular apparatus with the stages of the estrous cycle. Therefore, one is referred to the following reviews: Schwartz (1969), Everett (1972), Donovan (1972), and Bogdanove (1973). These authors discuss the evidence that exists concerning the control of the ovarian cycle by complex interactions among the brain, pituitary,
and ovary and the fact that any changes in these normal interactions will affect the ovary morphologically and functionally. But only hypothetically do these investigators mention the role of the perifollicular capillary bed with respect to ovarian function.

3. The General Vasculature of the Ovary

The dynamic characteristics of the general ovarian vasculature have been studied by many investigators utilizing a variety of techniques. Bassett (1943) was the first to study the vascular pattern in the ovary of the rat during the estrous cycle. He studied the ovarian vessels using injection techniques (carmine gelatin and india ink) as well as through direct observations in living animals (transillumination). The results of this study showed the random association of capillaries with primordial follicles, the development of a wreath of capillaries around the maturing follicles, and the invasion from the capillary wreath into the granulosa cell layer of the newly forming corpus luteum. In addition, he presented evidence about the cyclic changes of the vasculature which can be associated with follicular activity.

Delson et al. (1949) studied the vascular pattern in the human ovary using the vinyl acetate injection technique. He described the changes in the spiraling and
branching of the tertiary follicular vessels along with the variation in the degree of the spiraling and branching caused by ovarian pathology, the stage of follicular development, and corpus luteum formation. In addition the vasculature of the premature and infant ovaries was studied with regard to the spiral arteries. It was shown that the spiraling pattern was well developed from 7 months to full term in the human fetus. Although not presenting direct evidence, the authors suggest that maternal hormones may be producing the changes in the arterial patterns of the fetal ovaries. They also observed a regression of the spiral pattern 3 to 4 months after the infant's birth. It was concluded that the variations in the ovarian vasculature may be related to two factors: (i) adaptation to ovarian growth; (ii) to provide a mechanism for the regulation of the ovarian blood pressure. Reynolds (1950), using vinyl acetate injection techniques, observed changes occurring in the coils of the spiral arteries of the ovary. He concluded that changes occurred in the ovarian vasculature due to the necessity to adjust for: (i) follicular growth and regression; (ii) distribution of hormones; and (iii) regulation of ovarian blood pressure. Burr and Davies (1951), also using vinyl acetate, were concerned with vascular changes occurring in the rabbit
ovary. They observed the gradual engorgement of the ovarian blood vessels in conjunction with distension of the follicles. This in turn appeared to be associated with increased permeability of the vessels surrounding the follicle just prior to ovulation. The vascular pattern has also been studied in ovarian tumors. Omar et al. (1971), using the Gomori azo-alkaline phosphatase method to outline the capillaries, demonstrated the adjustment of the capillary pattern to the tumor present.

Therefore, the papers mentioned above demonstrate evidence about the dynamics of the ovarian vascular bed with respect to changes occurring in follicular growth, ovarian cyclicity, response to hormones, and pathology. The next factor to consider is the follicular apparatus and whether its permeability characteristics are changing or static.

4. Permeability of the Follicular Apparatus (Light and EM Studies)

The purpose of this section is to demonstrate that follicular permeability has been investigated to some extent, and that there are still unanswered questions regarding the proper understanding of differences that exist in follicular permeability.

Before reviewing the literature on macromolecular
passage to the cells of the mammalian follicle, it should be mentioned that this has been investigated in organisms with macrolecithal egg formation: e.g. Roth and Porter (1964) in the mosquito; Droller and Roth (1966) in the guppy; and Schechtman (1955) in the domestic fowl. They all demonstrate the passage of maternal macromolecules into the follicle and their incorporation into the yolk forming oocytes.

Of the papers dealing with molecular penetration into the follicles of the mammalian ovary, von Kaulla et al. (1958) demonstrated the passage of systemically injected, potentially noxious materials (Hg-mercapto-merin, $^{14}$C meprobamate, $^{131}$I human gamma-globulin) into the ovarian follicular fluid. The authors suggested that certain therapeutic agents may induce possible mutagenic effects, or have the capacity to interfere with the enzyme systems of the oocyte, especially when the drugs are administered over a long period of time or during the growth phase of oocyte development. Zacchariae (1958) observed a more rapid appearance of Evans blue (I.V. injection) in mature rabbit follicles just prior to ovulation, than in the less immature follicles. He believed this to indicate an increased permeability of the blood liquor barrier, which could be related to the mechanism of ovulation.
Glass (1961, 1963, 1966, 1971) conducted a series of investigations (mostly fluorescent antibody technique) to determine whether maternal proteins can enter the follicle of the mouse and be incorporated into the cells it contains. She has demonstrated the presence of systematically injected proteins in the oocyte (Glass, 1961), and further showed that the transfer appeared to be selective as to which species of serum molecules will enter the oocyte (Glass, 1966). She has further shown that the extent of molecular transport depends on the stage of follicular development (Glass and Cons, 1968). In addition Glass (1971) localized ovarian antigens in the oocyte that were similar or identical to particular mouse serum components. It was also found that nuclear localization of these antigens occurred at or just before oocyte growth occurred. Thus Glass suggested that the transferred serum molecule may have the effect of blocking the genetic readout during the storage of the egg before growth begins, because of a possible binding of the molecule with the gene complex. As a result this could affect oocyte growth.

In addition, transfer of circulating proteins to the follicle was also studied in the rat ovary (Mancini et al., 1963). The authors used fluorescent dye labelled
albumins and globulins to demonstrate that the injected molecules pass unchanged from the blood vessel into the follicles, and into the oocyte. Their observations of increased protein uptake by growing follicles led them to speculate that serum proteins might be a supplemental form of nutrition for the developing oocyte.

The above papers have demonstrated that serum proteins can enter the follicle and can be found in the oocyte, but this type of study does have its limitations. They were light microscopic investigations and did not have the resolving capacity to observe protein permeability of the follicular apparatus at the cellular level. As a result some investigators began to study the permeability of the follicular apparatus using the electron microscope along with certain tracer molecules.

Morris and Sass (1966) were the first to study the ultrastructure and permeability of the capillaries in the ovary of the rat and ewe. They used ferritin and india ink for their tracer work. But they did not specify in their publication whether the ferritin used was cadmium free (cadmium is toxic), and according to our standards the concentration of ferritin which they injected into each rat was rather high (250-500 mg). It should be evident that both of these factors could alter normal
capillary permeability and morphology. They (Morris and Sass, 1966) have emphasized the presence of large gaps found in the capillary endothelium of the corpus luteum. The gaps allowed free passage of the india ink and ferritin. The capillary bed of the perifollicular region was described as being less permeable than the capillaries of the corpus luteum. The abstract by Anderson (E. 1967) was the first to describe the uptake of HRP by the oocyte of the rabbit. The tracer was injected I.V. and all ovarian follicles were permeable to the tracer. Later Anderson (E. 1972) demonstrated the uptake of exogenous peroxidase by the follicles of the mouse ovary. The primary goal in these two studies was to observe the uptake of the tracer into the follicles and the oocyte. This was not correlated with phases of the estrous cycle, nor were the capillaries studied in order to observe whether the tracer left the capillary lumen through the interendothelial cell junctions or by vesicular transport.

Byskov (1969), in studying the preovulatory follicle of the mouse, observed gaps in the perifollicular capillary walls of the follicular apex just prior to ovulation. These gaps were similar to the type described by Morris and Sass (1966) in the capillaries of the rat corpus luteum, and by Carsten and Merker (1965) in the
capillaries of the rat vagina. Byskov suggested that gap formation in the capillaries may be induced by estrogen. The gap formation may also explain the increased follicular permeability to dyes observed just prior to ovulation in the rabbit (Burr and Davies, 1951) and in the rat (Bassett, 1943). In addition, Baccarini (1971) studied the ultrastructure of the perifollicular region in the rat ovary, and observed variations in the morphology of this region during the estrous cycle. These changes included alterations in the morphology of the basal lamina and the invasion of fibroblasts at the time of ovulation. The author speculated that this region may be hormone sensitive, thus causing the changes that were observed.

Most recently, Anderson (W. 1972) studied the permeability of perifollicular blood vessels and follicles in juvenile rat ovaries. Various tracers were used in the study: enzymatic and heme protein tracers (cytochrome C, HRP, hemoglobin); electron opaque protein tracer (ferritin) and non-protein tracers (thorotrast and carbon). The study compared the extent of molecular penetration from the perifollicular capillaries into the follicles. The cytochrome C and HRP were found to enter the follicles, while the basal lamina appeared to stop
the passage of ferritin and thorotrust into the follicle.

B. Capillary Structure and Function

An important component which may affect the transfer of systemic proteins into follicles is the capillary bed of the perifollicular region; thus it is important to consider its morphology. This will be approached by reviewing papers dealing with the capillary regarding the morphology and function of: (a) the junctions of the endothelial cells; (b) the plasmalemmal vesicles; and (c) the basal lamina of the capillary. These morphological characteristics will then be correlated with the function of capillary permeability.

The endothelial cell junction is an important component of the capillary wall because it is a region where macromolecules may be able to leave the capillary lumen. The intercellular junction of the capillaries of skeletal muscle have been described as having a zone of fusion of adjacent cell membranes within the luminal one-third of the intercellular cleft (Bruns and Palade, 1968). This area of tight junction is referred to as a zonula occludens. This zone may be considered impermeable and a continuous seal. However, Karnovsky (1968) found that HRP and lanthanum can pass through the junctions of skeletal muscle capillaries. Thus he concluded the cell junctions
consisted of maculae occludentes. This means that within these areas of cell membrane fusion, there are gaps (40 Å wide), rather than continuous sealed areas. It has now been shown that there are variations in the morphology of the endothelial cell junction depending on the type of tissue studied [Reese and Karnovsky (1967), capillaries of the brain; Shakib and Sunha-Vas (1966), retinal capillaries; and Areskog et al. (1964), heart capillaries]. But to date, there is not a complete classification of the types of endothelial cell junctions present in the capillary beds of the ovary.

Another important structure that should be mentioned when considering the transfer of macromolecules from the circulation into organs and individual cells is the plasmalemmal vesicle. They are somewhat unique features of the endothelial cells, although somewhat similar structures have been recognized in many other cells; e.g. in Kupffer cells (Parks and Peachy, 1955); in endothelial cells of hepatic sinusoids (Parks, 1956); in the amoeba (Schumaker, 1958; Nachmias and Marshall, 1961); and in the oocyte (Roth and Porter, 1964).

The morphology of the plasmalemmal vesicles has been described by Bruns and Palade (1968). The vesicles can be found free in the cytoplasm of the endothelium,
or open to the blood or tissue front. The median transit time for the plasmalemmal vesicle to traverse the endothelium is approximately one second (Casley-Smith and Clark, 1972). This vesicular transit does not appear to be unidirectional (Brandt, 1962; Brandt and Pappas, 1962; Wissig, 1964), a fact to be taken into consideration when studying macromolecular transport across the endothelial cell. The frequency of vesicles is highest in the attenuated periphery of the endothelial cell and least numerous in the perikaryon. Their possible role in transendothelial passage was a function first described by Palade (1953), but it was not yet determined whether this was an energy dependent process. Since then it has been suggested that the vesicles move randomly about the cell by thermodynamic bombardment of the surrounding molecules (Casley-Smith, 1963). In addition, it was shown that hypoxia, metabolic inhibitors, and cooling did not affect the number and morphology of the vesicles (Jennings and Florey, 1967). Therefore the actual movement of the vesicles does not require energy while vesicle formation by the cell membrane is an energy dependent phenomenon.

The final component of the capillary wall that may influence the passage of macromolecules from the lumen to the tissue front is the basal lamina surrounding the outer
surface of the endothelial cells. The basal lamina has been described as an acellular layer, 300-600 Å in thickness (Bruns and Palade, 1968). It stains as a PA-Schiff and PA-silver methenamine positive extracellular layer. Kefalides and Winzler (1966) analyzed the basal lamina material from the mammalian kidney and reported that the principal constituents include collagen and carbohydrates. This has been generally accepted as the primary makeup of the capillary basal lamina.

The selective filtering potential of the glomerular basal lamina has been studied using tracers; e.g. by Farquhar et al. (1961) and Farquhar and Palade (1960) showing that it retains molecules the size of ferritin; and by Graham and Karnovsky (1966) showing that it did not retain HRP.

C. Use of Tracers in Permeability Studies

This section will review the literature with the goal of explaining the reasons for choosing ferritin and horseradish peroxidase (HRP) as tracers in this study.

Horseradish peroxidase is a hemoprotein, crystallized from the horseradish plant (Paul, 1963). The horseradish peroxidase is extracted from the roots of the wild horseradish plant (Cochlearia armoracia L.). There are several methods (electrophoresis and fractionation
procedures) used to extract the HRP. For example, one method involves: maceration of the plant, removal of most inactive material from the crude extract by precipitation with a mixture of chloroform and ethanol, and fractional precipitation with ammonium sulphate and ethanol (Kenten and Mann, 1954). The product obtained is a stable dry preparation which can be crystallized from the ammonium sulphate solution. The weight of this hemoprotein has been determined to be approximately 40,000 daltons (Keilin and Hartree, 1951) and it has an estimated radius of approximately 25-30 Å (Karnovsky, 1967). The tracer does not have an inherent electron opacity; therefore the visualization of this tracer under the electron microscope is the result of a reaction product formed by incubation with a substrate (Karnovsky, 1967). (More details of the reaction will be described in the materials and methods section). The tracer does not appear to alter the capillary permeability in mice (Cotran and Karnovsky, 1967; Cotran et al., 1968). When injected into the systemic circulation, the tracer is not altered by enzymes in the circulation nor is there significant binding of the tracer to plasma proteins or polymerization of the tracer (Vegge et al., 1971).

HRP has been successfully utilized in many
experiments aimed at resolving the ultrastructural basis of permeability; e.g. by Graham and Karnovsky (1966) studying the proximal tubules of the mouse kidney; by Karnovsky (1967) working on the capillaries of skeletal muscle; by Becker et al. (1967) observing the choroid plexus in the rat; by Enders and Wimsatt (1971) studying the chorioallantoic placenta of the bat; in the guinea pig chorioallantoic placenta (King and Enders, 1971); by King (1971) in the guinea pig parietal yolk sac; by Clementi (1970) observing lung capillaries of the mouse; by Cotran and Karnovsky (1968) studying the mouse mesothelium; by Leak (1972) investigating the blood-tissue-lymph interface; by Fawcett et al. (1970) localizing the blood testis barrier; by Strauss (1972) studying different segments of the nephron; by Graham et al. (1969) studying the mouse liver; and by Venkatachalam et al. (1970) determining glomerular permeability.

Ferritin is a protein molecule that contains up to 23% iron in its dry weight. Laufberger (1937) was the first to isolate ferritin from the horse spleen. The technique involved producing an aqueous spleen extract, precipitating with ammonium sulfate, and finally crystallizing out the ferritin with cadmium sulfate. The procedure has been carried further to remove the
toxic cadmium sulfate (Farrant, 1954). He washed the crystals in distilled water and dialyzed the solution in order to have a cadmium free ferritin product. It has a normal physiological function of iron storage in different parts of the body (e.g. liver, spleen) (Farrant, 1954). The ferritin molecule has an inner core diameter of approximately 55 A (Farrant, 1954) of ferric hydroxide micelles, surrounded by a spherical shell of protein which has a molecular weight of 462,000. The overall diameter of the molecule is approximately 110 A (Harrison, 1959). Ferritin gets its inherent electron opacity from the 55 A ferric micellar core. This protein possesses certain favorable characteristics making it suitable for tracer work: e.g. (1) its molecules are relatively similar in size, with only a small portion of multiples of monomers being present (Harrison and Gregory, 1965); (2) it falls within the size range of most plasma proteins (65x90 A for albumin, 60x150 A for γG-globulin, 200 A for γ2 Macroglobulin, and 200x300 A for 195 γM globulin as determined by Hoglund and Levine (1965); and 200x40 A for γglobulin as was determined by Hall et al. (1959); (3) if it is cadmium free, it is well tolerated by the experimental animals; and (4) it is retained in the vascular system in high concentrations for at least 24 hours.
Ferritin has been used successfully in many permeability experiments; e.g. by Farquhar et al. (1961) studying the glomerular capillary wall; by Mounsbach (1966) observing the proximal tubules of the rat; by Clementi and Palade (1969) investigating the intestinal capillaries of the mouse; by Bruns and Palade (1968) studying the capillaries of skeletal muscle; by Fawcett et al. (1970) localizing the blood-testis barrier; by Fedorka et al. (1971) studying the mesothelial cells of the mouse omentum; and by King (1972) observing the guinea pig parietal yolk sac.

Therefore it can be seen that ferritin and HRP are tested tracers which exhibit the reliability needed in order to be used for studies on capillary permeability.

D. Influence of Hormones on Capillary and Ovarian Morphology

Since this thesis also deals with the theory of permeability changes in the ovarian capillaries during the estrous cycle, this next section of the literature review will be devoted to presenting some evidence that serum hormone levels can affect general vasculature, capillary permeability, and the morphology of the ovary.

Wurtman (1964), using the $^{42}\text{K}$ uptake method, found
that the LH hormone caused an increase in the fractional perfusion of the rat ovary. Wurtman also found that the fractional perfusion of the rat ovary was highest during the estrous stage of the estrous cycle. To correlate the results of the high perfusion rate at estrus with his single injections of LH, Wurtman suggested that the endogenously released LH operating over a period of hours during proestrus produces an ovarian vascular response with a different time effect from that associated with the single large injection of LH.

Szego (1965) observed a histamine release and histamine dissipation from the ovary in response to the intravenous injection of LH. Szego correlates the results with the known effect of histamine on the microvasculature (hyperemia) inducing vascular responses which may produce alterations in the availability of metabolites to the target cells. Lipner (1971) studied the effect of a histamine releasing drug (compound 48/80) and an antihistamine (diphenhydramine) on the histamine concentration in ovarian tissue and the histamine concentration in the blood of PMS-HCG primed rats. He found that the drugs caused an inhibition in the ovarian weight which would be normally produced by the hyperemia reaction from HCG. The treatment reduced the number of ova shed,
although all the rats ovulated. Thus Lipner concluded that histamine plays a major role in the increase in ovarian weight but not in ovulation.

Wolff et al. (1967) demonstrated changes in the ultrastructure of the capillaries in the rat vagina during the proestrus and estrus stages of the sexual cycle. The capillaries developed a porous morphology during these stages as opposed to the continuous endothelial lining at the metestrus and diestrus stages. They were also able to induce this gap morphology in the endothelium after estrogen treatment in castrated animals. The gaps observed between endothelial cells were similar to those found in the capillaries of the corpus luteum of the rat (Morris and Sass, 1966). Piacsek and Huth (1971) observed a significant increase in blood flow through the ovary due to LH injection. It should also be mentioned that this increase due to LH administration was not seen in the femoral vein, indicating that the histamine releasing activity of LH appears to be restricted to ovarian tissue. Forbes and Glassen (1972) placed steroid pellets in the uterine horn of mice and observed a dilatation of the ovarian vein. Maqueo et al. (1972) observed changes in ovarian morphology after use of steroid contraceptive agents, and Moghissi (1972) observed morphological
changes in the corpus luteum of women treated with continuous micro-doses of progestogens.

The intimate relationship between the blood supply of a tissue and its level of function has been long recognized. Thus, the microcirculation, with its variable degree of expansion and permeability, offers a critical site for the imposition of regulatory influences on the transport of substances upon which the function of the target cell depends.

As a result of this review it can be seen that ovarian morphology and ovarian circulation are constantly changing in an orderly pattern which has been correlated with the estrous cycle. Further, that there is a need to study the morphology of this perifollicular capillary bed because it may influence the passage of circulating proteins into the growing follicles of the ovary.
III. MATERIALS AND METHODS

A. The Experimental Animal

The female non-inbred Swiss albino mouse [SCH:ARS HA(ICR) strain] was used as the experimental animal for the described experiments. The mice were purchased from ARS/Sprague-Dawley, Madison, Wisconsin. They were received at 3 weeks of age, in a weight range of 11 grams to 13 grams. Upon arrival the animals were housed individually in plastic cages at the animal research facility of Loyola Medical Center. The room temperature was approximately 24°C. Purina Lab Chow and water were given ad libitum. The light cycle was 10 hours dark and 14 hours light (lights on 05:00 to 19:00 hours).

1. Part of the investigation required the use of sexually immature, non-cycling females. The animals were approximately 4 weeks old, with a weight range of 17 grams to 20 grams. Only animals with an unopened vagina and no corpora lutea were used.

For other investigations requiring mature cycling females, mice 6-7 weeks old were used. Vaginal smears were taken at 08:00 and 17:00 hours. The smears were examined in unstained wet preparations. Identification
of cycle stage of the animal was according to the descriptions of Allen (1922) and Bingel and Schwartz (1969). The stage of the cycle was established by vaginal smear then subsequently confirmed by the uterine wet weight, and the appearance of the oviducts. Only animals that completed two consecutive 4 or 5 day cycles were used in the experiments.

2. Whenever a group of mice were put in the cages, there was a complete lack of a sexual cycle in the animals. But when the mice were housed individually, approximately half the mice exhibited a regular cycle. Only animals in proestrus and metestrus were chosen for the experiments. Any animal not meeting the requirements described below for cycle determination was not accepted.

The proestrous animal had to exhibit the typical vaginal smear (nucleated, cornified epithelial cells, and some leucocytes, depending on the cycle length), swelling of the external genitalia, and a characteristic uterine weight.

The metestrous animal had to exhibit estrus 24 hours preceding the experiment, had to show a typical vaginal smear (primarily leucocytes with some nucleated and cornified epithelial cells), and a characteristic uterine weight. Tables 3, 4, and 5 present the data on
the cycling animals.

Experiments were terminated by decapitation at 18:00-19:00 hours.

B. Studies on Normal Ovarian Morphology

This segment was devoted to studying the normal morphology of the mouse ovarian follicles along with the associated perifollicular regions. Emphasis was placed on the morphology of the perifollicular capillary bed in order to determine whether there are any differences in the microvasculature when comparing the immature, proestrous, and metestrous animals. Also it was intended to compare these observations with previous investigations dealing with the normal morphology of this tissue (Peters, 1969; Byskov, 1969; Odor and Blandau, 1969; Stegner and Onken, 1971; and Chiguoine, 1960).

Cycling and non-cycling animals were used for this study. The mice were decapitated, the abdomen was opened, and the ovaries and uterus immediately removed. The ovaries of the non-cycling mice were examined to ascertain the absence of corpora lutea. The stage of the cycle for the mature mice was determined as previously described.

1. Fixation, Dehydration, and Embedding

The ovaries were immediately immersed in 3.125% aqueous Glutaraldehyde solution (Sabatini et al., 1962;
1963) buffered to pH 7.4 with 0.2 M Sorensen's phosphate buffer (Hayat, 1972), chilled at 4°C. The fixation time was one and a half hours. After the prefixation the ovaries were further dissected under a dissecting microscope to isolate selected areas. After three, one-hour washes in Sorensen's phosphate buffer, the tissue was post-fixed for one and a half hours in phosphate buffered 1% osmium tetroxide (Ledbetter and Porter, 1963). The tissue was then dehydrated in increasing concentrations of chilled ethyl alcohol at ten minute intervals each. Then the tissues were passed through two, ten-minute changes of propylene oxide at room temperature. Finally the tissue was gradually infiltrated with epon and embedded in Epon 812 (Luft, 1961).

The epon embedded blocks of tissue were polymerized for 48 hours in a 60°C oven. The blocks were sectioned with glass knives (Latta and Hartman, 1950) on a Reichert OmU2 ultramicrotome. The sections were placed on uncoated 200 mesh copper grids and either "stained" with uranyl acetate (Swift and Rasch, 1958) for enhanced contrast in electron opacity, or left "unstained." The sections were examined on a RCA EMU 3F-2 electron microscope at 50 kV. The original magnifications used on the microscope ranged from X1,400 to X16,000.
C. Scanning Electron Microscopy

The ovaries from two mice were prepared for viewing with the scanning electron microscope (SEM). The tissues were prepared identically with those for transmission electron microscopy (TEM), except that infiltration and embedding in epon were omitted. The ovarian fragments were dried in a vacuum dessicator. The specimens were mounted on specially engineered aluminum chucks, and coated with a 200-400 Å layer of gold. The tissue was then observed with a SEM (Advanced Metals Research, Model 900) courtesy of Standard Oil Research.

D. Horseradish Peroxidase Experiments

In this study non-cycling sexually immature rats were used, along with sexually mature mice.

1. Injection of HRP

The mice were lightly anesthetized with an 0.25 ml I.P. injection of pentobarbital sodium (4.3 mg/ml). The tracer (HRP, type II, Sigman Chemical Co.) was dissolved in a normal saline solution. This was injected through the tail vein using a tuberculin syringe with a Hubner needle (27 G).

Originally the test studies were conducted using varying amounts of HRP (9.0 mg, 5.0 mg, 1.7 mg, and 1.0 mg), along with different volumes of normal saline (0.6
ml, 0.5 ml, 0.2 ml, and 0.1 ml), in an effort to determine the ideal concentration of HRP saline volume which showed consistent reaction product and did not alter the normal capillary permeability, as too high a concentration of HRP will affect capillary permeability (Clementi, 1970). The mice were decapitated at various time intervals after the injection of the tracer (30 seconds to 30 minutes). The controls consisted of: (1) HRP and no incubation; (2) incubation but no HRP; (3) HRP but hydrogen peroxide lacking in the incubation medium; and (4) HRP but no diaminobenzidine in the incubation medium. The experimental variations and controls are indicated in Table 1.

2. The HRP Technique (Karnovsky, 1967)
   1. Animals decapitated;
   2. Fixation with phosphate buffered glutaraldehyde for one hour, then wash in buffer as described previously;
   3. Cut frozen sections (40-80 microns) of the ovary;
   4. Sections incubated in the medium, at room temperature for 30 to 60 minutes (Graham and Karnovsky, 1966; Karnovsky, 1967);

   Incubation Medium:
5.0 mg 3,3' Diaminobenzidine tetrahydrochloride (DAB), Sigma Chemical Co.; 10.0 ml 0.05 M Tris-HCl buffer pH 7.6; 0.1 ml 1% H₂O₂ (freshly made from 30% H₂O₂).

5. The tissues subsequently post-fixed in 1% osmium tetroxide for one hour;

6. Dehydration, infiltration, and embedding was done as previously described.

The reaction product was visualized under the light microscope as having a brownish black color in toluidine blue stained sections (1 micron) of epon embedded tissue. Under the electron microscope the reaction product appeared as an electron dense area.

In the peroxidase reaction the DAB acts as an electron donor, and is oxidized at the site of the enzyme activity to a highly insoluble, brown, polymeric compound. On post-fixation with osmium tetroxide, the reaction product reacts avidly with the osmium, yielding a black, insoluble, non-crystalline deposit which is highly electron opaque (Karnovsky, 1968). This technique gives a fine and sharp localization at the ultrastructural level.

The peroxidase injected tissue was examined with the purpose of: (1) describing the passage of this tracer from the capillary lumen into the follicle; (2)
determining the rate of passage in conjunction with different circulating times; (3) determining any variability in the tracer passage; and (4) observing cellular uptake.

E. Ferritin Experiments

Immature and mature mice were used in this series of experiments. Tables 2, 3, and 4 present the data on this series of studies.

1. Injection Techniques

Animals were lightly anesthetized with an I.P. injection of pentobarbital sodium. Each mouse received an injection into the tail vein (27 g Hubner needle) of an isotonic saline solution containing the ferritin. The ferritin is a 6X crystallized, lyophilized, cadmium free, B grade protein purchased from Calbiochem, California.

Variation in the experiment consisted of changing the volume of saline and injecting saline alone.

2. Tissue Preparation for E.M.

After the tracer was allowed to circulate for a specified time interval (2 minutes to 12 hours), the animals were decapitated. One ovary from each mouse was processed for electron microscopy by the method previously described. No incubation is needed to visualize the ferritin. The ferritin may be seen under the electron microscope as electron opaque particles having an
approximate diameter of 55 Å (Ainsworth and Karnovsky, 1971).

3. Tissue Preparation for Light Microscopy

The other ovary from each animal was processed for light microscopy utilizing the paraffin embedding technique.

1. The tissue was fixed in buffered formalin;
   1000.0 ml 10% formalin
   4.0 gm sodium acid phosphate
   6.5 gm anhydrous disodium phosphate
2. Dehydrated in a graded series of ethyl alcohol;
3. Cleared in benzene;
4. Infiltrated and blocked in Tissue Prep (Fisher Scientific Co., melting point of 52.5°C ± 0.5°C);
5. Sectioned tissue at 15 to 40 microns.

4. Staining of Ferritin for Light Microscopy

Perl's method for ferric iron (Pearse, 1961) was used:

1. Hydrate sections to distilled water;
2. Expose the sections to a fresh mixture of equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid for 30 to 60 minutes;
3. Wash in distilled water;
4. Counterstain nuclei with 1% aqueous, neutral
red for 3 minutes;

5. Wash, dehydrate in alcohol, clear in xylene, and mount in synthetic resin.

The results will show the ferric iron of ferritin as a deep prussian blue, and the nuclei will be red.

5. Staining Ferritin for E.M.

Because of the small size of the ferritin iron core, Bismuth Subnitrate was used to intensify the electron opacity of the ferritin (Ainsworth and Karnovsky, 1971). The following method was used:

Dissolve 400 mg sodium tartrate in 10 ml of 2N sodium hydroxide. This solution is added dropwise to 200 mg of bismuth subnitrate while being stirred (by a magnetic bar). Addition of all of the sodium tartrate chelates all of the bismuth and the final solution should be clear. This stock solution is diluted 1:50 with distilled water. The grids are placed on a few drops of the working solution at room temperature for 30 to 60 minutes. The grids are then washed in distilled water. This stain enhances the electron opacity of the ferritin and adds some contrast to the tissue.

The tissue from the ferritin injected animals was examined in order to: (1) observe the passage of the tracer from the perifollicular capillary lumen into the
fOLLICLE; (2) determine the rate of passage with respect to the different circulation time of the tracer; (3) determine the possible sites which might restrict ferritin passage; and (4) observe cellular uptake of the tracer.

6. Other Staining Procedures
   a. Alkaline toluidine blue stain was used for staining 1 micron epon sections for light microscopy (Trump et al., 1961);
   b. Some paraffin embedded tissue was stained with the routine hematoxylin and eosin staining method.

7. Method for Semiquantifying the Penetration of Ferritin
   a. Magnification of the electron microscope was calibrated with a carbon replicating grid (28,800 lines per inch);
   b. Photomicrographs of known magnification were used;
   c. 15 to 20 photomicrographs were randomly chosen from the 4 animals used in each of the following experimental groups: (1) immature animals - 2 min, 5 min, 15 min, and 1 hour; (2) proestrus animals - 2 min and 5 min; (3) metestrus animals - 2 min and 5 min; and (4) one immature animal at 5 min. Thus a total of 33 animals were used and a total of 162 photomicrographs evaluated;
   d. Surface areas on the photographs were measured
using a Keuffel and Esser compensating polar planimeter. Random areas were measured in the capillary lumen and in the pericapillary region;

e. The ferritin molecules were counted in each area measured, and calculated to a value corresponding to the number of molecules per square micron;

f. The results from the luminal counts and pericapillary counts were converted to a value (out/in) which is the percentage of ferritin in the perifollicular region.
TABLE 1

MICE INJECTED WITH HORSERADISH PEROXIDASE
(Immature Mice)

<table>
<thead>
<tr>
<th>HRP (mg)</th>
<th>SALINE (cc)</th>
<th>CIRCULATION TIME</th>
<th>INCUBATION MEDIUM</th>
<th>NO. OF ANIMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>0.6</td>
<td>10 min</td>
<td>Complete</td>
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<tr>
<td>5.0</td>
<td>0.5</td>
<td>15 min</td>
<td>&quot;</td>
<td>2</td>
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<tr>
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<td>0.5</td>
<td>15 min</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>5.0</td>
<td>0.5</td>
<td>15 min</td>
<td>No H$_2$O$_2$</td>
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</tr>
<tr>
<td>5.0</td>
<td>0.5</td>
<td>15 min</td>
<td>No DAB</td>
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</tr>
<tr>
<td>9.0</td>
<td>0.6</td>
<td>30 min</td>
<td>Complete</td>
<td>2</td>
</tr>
<tr>
<td>1.7</td>
<td>0.3</td>
<td>1 min</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>1.7*</td>
<td>0.3</td>
<td>2 min</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>1.7**</td>
<td>0.3</td>
<td>5 min</td>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>1.7</td>
<td>0.3</td>
<td>15 min</td>
<td>&quot;</td>
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</tr>
<tr>
<td>1.7</td>
<td>0.2</td>
<td>30 sec</td>
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</tr>
<tr>
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<td>0.2</td>
<td>30 sec</td>
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<td>0.2</td>
<td>45 sec</td>
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</tr>
<tr>
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<td>&quot;</td>
<td>1</td>
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<tr>
<td>1.0</td>
<td>0.1</td>
<td>1 min</td>
<td>&quot;</td>
<td>1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>5 min</td>
<td>&quot;</td>
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*Sexually mature Metestrous Mice
**Sexually mature Proestrous Mice
<table>
<thead>
<tr>
<th>FERRITIN (mg)</th>
<th>SALINE (cc)</th>
<th>CIRCULATION TIME</th>
<th>NO. OF ANIMALS</th>
</tr>
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<tbody>
<tr>
<td>30.0</td>
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<td>2 min</td>
<td>1</td>
</tr>
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<tr>
<td>90.0</td>
<td>0.4</td>
<td>5 min</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>0.3</td>
<td>2 min</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>0.3</td>
<td>2 min</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>0.3</td>
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<td>50.0</td>
<td>0.3</td>
<td>15 min</td>
<td>3</td>
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<td>50.0</td>
<td>0.3</td>
<td>60 min</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>0.3</td>
<td>6 hrs</td>
<td>3</td>
</tr>
<tr>
<td>50.0</td>
<td>0.3</td>
<td>12 hrs</td>
<td>2</td>
</tr>
</tbody>
</table>
### TABLE 3
PROESTROUS MICE INJECTED WITH 50 MG OF FERRITIN
(Data from individual animals)

<table>
<thead>
<tr>
<th>BODY WT. (gms)</th>
<th>UTERINE WT. (mg)</th>
<th>SALINE (cc)</th>
<th>CIRCULATION TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.6</td>
<td>175.7</td>
<td>0.2</td>
<td>2 min</td>
</tr>
<tr>
<td>23.4</td>
<td>195.1</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>20.9</td>
<td>170.1</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>21.3</td>
<td>180.2</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>22.7</td>
<td>187.6</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>21.6</td>
<td>172.4</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>22.1</td>
<td>152.2</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>22.6</td>
<td>169.1</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>23.0</td>
<td>192.0</td>
<td>No Injection</td>
<td>0</td>
</tr>
<tr>
<td>23.7</td>
<td>190.4</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>21.4</td>
<td>161.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>22.4</td>
<td>182.5</td>
<td>&quot;</td>
<td>0</td>
</tr>
</tbody>
</table>

**SALINE INJECTION WITHOUT FERRITIN**

| 22.4 | 179.5 | 0.2 | 2 min |
| 22.8 | 170.2 | "   | "     |
| 21.5 | 187.4 | "   | "     |
| 23.6 | 201.2 | "   | "     |
### TABLE 4

**METESTROUS MICE INJECTED WITH 50 MG OF FERRITIN**  
(Data from individual animals)

<table>
<thead>
<tr>
<th>BODY WT. (gms)</th>
<th>UTERINE WT. (mg)</th>
<th>SALINE (cc)</th>
<th>CIRCULATION TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.0</td>
<td>79.0</td>
<td>0.2</td>
<td>5 min</td>
</tr>
<tr>
<td>20.3</td>
<td>68.8</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>22.1</td>
<td>74.4</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>23.5</td>
<td>77.3</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>24.0</td>
<td>84.4</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>21.5</td>
<td>82.5</td>
<td>&quot;</td>
<td>2 min</td>
</tr>
<tr>
<td>22.9</td>
<td>69.5</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
<tr>
<td>22.1</td>
<td>74.8</td>
<td>&quot;</td>
<td>5 min</td>
</tr>
</tbody>
</table>

**NON-INJECTED CONTROL**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tr>
<td>21.2</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23.1</td>
<td>104.0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>20.7</td>
<td>71.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23.9</td>
<td>94.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**SALINE INJECTED CONTROL**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>23.0</td>
<td>89.7</td>
<td>0.2</td>
<td>2 min</td>
</tr>
<tr>
<td>23.3</td>
<td>93.5</td>
<td>0.2</td>
<td>2 min</td>
</tr>
<tr>
<td>21.9</td>
<td>73.6</td>
<td>0.2</td>
<td>2 min</td>
</tr>
<tr>
<td>22.7</td>
<td>82.4</td>
<td>0.2</td>
<td>2 min</td>
</tr>
</tbody>
</table>
## TABLE 5

**STATISTICAL DATA ON THE CYCLING MICE**

### PROESTROUS MICE

<table>
<thead>
<tr>
<th>Uterine Wt. mgs</th>
<th>Mean</th>
<th>179.51 ± 12.9 S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Wt. gms</td>
<td>Mean</td>
<td>22.32 ± .86 S.D.</td>
</tr>
</tbody>
</table>

### METESTROUS MICE

<table>
<thead>
<tr>
<th>Uterine Wt. mgs</th>
<th>Mean</th>
<th>84.04 ± 11.55 S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Wt. gms</td>
<td>Mean</td>
<td>22.23 ± 1.08 S.D.</td>
</tr>
</tbody>
</table>
IV. RESULTS

The general histology of the ovary is illustrated in Figure 3. The types of follicles studied ranged from the early unilaminar, to the mature Graafian follicle. Atretic follicles were not studied with respect to tracer penetration.

A. Follicular Apparatus

Because this thesis is concerned with the passage of protein tracers from the systemic circulation into the ovarian follicle, a description of the region where the protein passage occurs will be presented. In addition, the follicular apparatus of the immature and cyclic mice will be compared.

The general relationship of the capillary bed to the follicle is demonstrated in Figure 4. The theca interna and externa may be present or absent, depending on the stage of follicular development. Capillaries are found next to or a few cell layers removed from the basal lamina of the follicle, situated in the connective tissue in each of the follicles studied (Figure 4). The portion of the follicle inside of the basal lamina is always avascular.
In order for macromolecules to reach the ovarian follicles and the cells it contains, a number of barriers have to be penetrated. The first of these is the cells lining the capillary wall. The capillary wall was found to consist of a single layer of continuous endothelium. There were no large gaps or pores present between the endothelial cells, regardless of the stage of follicular development or the reproductive state of the animal.

The general size and shape of the endothelial cell could not be determined in this study. However, in cross section, the endothelial cells appeared to be approximately three microns thick in the nuclear region and flattened toward the periphery to approximately 0.4 microns or less (Figures 5 and 6). The nuclear region bulged into the lumen of the capillary.

The general cellular organelles found in the cytoplasm of the endothelial cell were typical with regard to location and morphology. The perinuclear region of the endothelial cell contained the Golgi complex, rough and smooth endoplasmic reticulum, mitochondria, glycogen, and some plasmalemmal vesicles. The thinner regions of the endothelial cell possessed a few mitochondria, free ribosomes, some elements of endoplasmic reticulum, and many plasmalemmal vesicles.
The plasmalemmal vesicles of the endothelial cell were often found either free in the cytoplasm or attached to the cell membrane, open either to the lumen or to the tissue front. Some of the vesicles were attached to the cell membrane by a narrow neck, while others were lacking this feature. All the vesicles were delineated by a membrane which appeared identical to the plasmalemmal of the endothelial cell. The contents of the vesicles varied slightly in electron density, sometimes matching the material that filled the pericapillary spaces, at other times similar to the density of the plasma in the capillary lumen. The vesicles were most frequent in the narrower segments of the endothelial cell, while fewer were found in the nuclear region and the cell junctions. The size of the vesicle varied widely, probably resulting from the plane of sectioning.

It should also be mentioned that similar vesicles were found in other cell types in the vicinity of the capillaries; e.g. pericytes, thecal cells, and smooth muscle cells.

The endothelial cell junctions varied somewhat in their morphology in parallel with the width of the cell. When the endothelial cell wall was thicker there was a tendency for an overlapping, interdigitating type of
cellular contact, while if the cell wall was thinner there was a tendency for the blunt type of abutments to be more prevalent. At lower magnifications the junctions appear to have regions of complete fusion (Figure 5). However at higher magnifications, the interendothelial cell clefts appeared to have intermittent points where the two membranes almost came into contact, but not completely. The morphological pattern of the endothelial junction did not vary with respect to the different phases of the estrous cycle or the stage of follicular development.

The tissue front (contra luminal) of the endothelial cell is completely surrounded by a basal lamina. This extracellular layer has an approximate thickness of 500 Å. Its morphological appearance (in sections stained with uranyl acetate) demonstrates an electron density greater than that observed in the intercellular spaces between the cells of the pericapillary region.

Occasionally pericytes are found adjacent to the basal lamina which surrounds the pericyte. The cyto logical characteristics of the pericyte includes a fibroblast type of shape, an abundance of dense cytoplasmic granules presumed to be glycogen, a large number of mitochondria, rough and smooth endoplasmic reticulum,
and a large nucleus. Their morphological association with the capillary wall does not appear to be a barrier to macromolecular passage.

The next region through which the tracers must pass is the intercellular space between cells surrounding the unilaminar follicles, or the intercellular spaces of the thecal layer in the case of the more mature follicles (Figures 4-7). It is a matrix having less electron density than the basal lamina (in uranyl acetate stained sections).

The next region for the passage of the macromolecules would be the basal lamina surrounding the follicles (Figures 5 and 6). This acellular material is an amorphous, continuous layer of moderately electron dense material adjacent to the outer layer of granulosa cells. It was found surrounding the follicles in all stages of its development. The ultrastructural appearance of this acellular layer did not vary with respect to the different stages of follicular development. The only exception was in the atretic follicles, where there was an abundance of collagen formation.

There are two possible pathways for the macromolecules after passing through the basal lamina of the follicle. The first is through the cytoplasm of
the granulosa cells. Vesicle formation could be observed on the plasmalemma of the granulosa cells suggesting uptake of materials by these cells. The second pathway is through the intercellular spaces of the granulosa layer. In the unilaminar follicle this space was small because of the close apposition of the follicle cells, especially those in the periphery. However, as the follicles develop an antrum the intercellular spaces between the granulosa cells became larger (Figure 7).

In order to reach the oocyte, the macromolecules must next pass through the zona pellucida. The zona may be present, forming (Figure 8), or absent, depending on the stage of follicular development. The zona appeared to be an amorphous layer similar in composition to the follicular fluid only more electron dense.

Pinocytotic vesicles could be observed forming on the oolema, indicating that some type of endocytosis or exocytosis was taking place. The content of the vesicles had a similar electron density to the vesicles observed in the endothelial cells, pericytes, and granulosa cells.

The above observations on the follicular apparatus were made for the following reasons: (1) to determine the "normal" morphology of the region where
macromolecular passage from the capillary lumen to the oocyte will occur; (2) to determine whether any morphological changes occur in this region during the estrous cycle; and (3) to use this morphological information as a control for the tracer experiments.

B. Horseradish Peroxidase Experiments

1. Control Studies

The experiments in this section were conducted in order to determine whether there was any endogenous peroxidase activity present in the ovary, and whether there was any alteration in morphology caused by the incubation technique.

Tissue from animals injected with saline only, but incubated in a complete medium, is shown in Figures 9 and 10. This tissue demonstrates that the incubation technique did not alter the morphology of the tissue. The oocyte and granulosa cells do not demonstrate any endogenous peroxidase activity. The capillary wall and lumen do not show peroxidase positive reaction. The red blood cells do stain intensely black in the complete medium (Figure 10). The intercellular spaces of the thecal layer, and the basal lamina of the capillary and follicles lack the reaction product.

No reaction product could be found in any part of
the follicular apparatus when the DAB or \( \text{H}_2\text{O}_2 \) were absent from the incubation medium.

2. HRP Pilot Experiments

Pilot experiments were conducted in order to determine the reliability of the HRP technique.

Various doses (1.0-9.0 mg) of HRP were injected into the animals (14-20 gm). This was done in order to determine the lowest dosage of HRP that could be used and still give a consistent reaction product after incubation with the substrate. In addition, it was important to determine whether variation in the concentration of HRP injected would affect the rate of HRP leaving the capillary. An HRP dose in the range of 1.7 mg to 1.0 mg per 14-20 gm mouse gave a reaction product which was reproducible. The amount of saline injected (0.1 ml to 0.5 ml) did not alter capillary morphology or the rate of HRP leaving the capillary lumen. Incubation of the HRP injected tissue for one hour produced a reaction product which was consistent throughout the tissue section.

3. HRP Experimental Results (Summarized in Table 6)

The tracer could be found in the intercellular spaces adjacent to the follicle and within the follicles as rapidly as 30 seconds after the HRP injection (Figure
ll, electron dense regions indicated by arrows). This indicates a rapid passage of the tracer from the capillary lumen.

The HRP was observed to leave the capillaries in two ways: (1) through the intercellular spaces of the endothelium (Figure 12); and (2) in the plasmalemmal vesicles (Figure 13). The entire endothelial cell junction is filled with the reaction product. This appeared to be the primary pathway for the exit of HRP from the capillary, since vesicular transport could not account for the rapid exit of this tracer from the capillary.

The basal lamina surrounding the capillaries along with the intercellular spaces of the pericapillary region do not seem to restrict the passage of this tracer. The cells in the region eventually take up the tracer after longer circulation times for the tracer (2 minutes).

The basal lamina of the follicle, the intercellular spaces between the granulosa cells, and between the granulosa cells and oocyte, were permeable to the tracer in all the experiments (Figure 14-18). This was also true for the zona pellucida (Figure 18).

After longer circulation times for the tracer, the peroxidase reaction product became more electron
dense in the intercellular spaces and zona pellucida of the follicle (1 min to 30 min). There was also greater cellular uptake. Figure 14 illustrates the presence of HRP in the membrane bound vesicle of a granulosa cell. The larger vesicles usually have an electron dense reaction product in the periphery of the vesicle and a less dense central region. The smaller vesicles appear uniformly electron dense.

The tracer was also observed in some oocytes. Figure 15 demonstrates the presence of HRP in a vesicle along with an example of a vesicle forming which contains the reaction product. HRP was often found in the oocytes of the younger follicles (unilaminar and early antrum formation) but rarely found in the oocytes of the more mature follicles.

The observation on the passage of HRP from the systemic circulation into the follicle was the same in the immature and mature animals. The entire follicular apparatus is freely permeable to the HRP. The only variation observed was in the case of greater cellular uptake by the oocytes of the younger follicles.

C. Ferritin Experiments on Sexually Immature Mice

1. Pilot Experiments

The first series of experiments dealt with
determining the dose of ferritin and the volume of saline to be injected. The volume (0.5 to 0.2 ml) of saline injected did not alter the rate of ferritin leaving the capillary. When a high concentration of ferritin was used (90 mg/0.5 ml), there were examples of abnormal leakage of the tracer from the venules (Figure 19). This was not observed when lower concentrations of ferritin were injected (30 to 50 mg). Vascular leakage refers to abnormal gaps in the endothelium which allow a free passage of the tracer into the pericapillary spaces.

2. Controls (Figures 20-21)

These experiments demonstrated the absence of any electron opaque particles which might be interpreted as ferritin molecules. (It should be mentioned that both the control tissue and the ferritin injected tissue were stained either with bismuth subnitrate or "unstained.")

3. Ferritin Experiments on Sexually Immature Mice

One must use high source magnification (at least X16,000) with the electron microscope in order to observe the ferritin molecules, especially when the tracer is present at low concentration. For example, Figure 22 is a low magnification electron micrograph of the type of capillary studied. The ferritin is visible in the capillary lumen, but it is impossible to observe the low
concentration of the tracer present in the intercellular spaces.

The way in which the ferritin leaves the capillary is different from the HRP results. Regardless of the stage of follicular development, the tracer was never found in the tight junctions of the endothelial cells (Figures 23-25). The only observable means for the transcapillary passage of ferritin is by vesicular transport (Figure 26). The vesicles were not equally labelled with the tracer (range 0 to 4 molecules). The probable reason for this variation in vesicular content is that the plane of sectioning through the vesicle may have missed its ferritin content (median diameter of a vesicle is approximately 700 Å while the diameter of a ferritin molecule is approximately 110 Å).

The passage of ferritin from the systemic circulation into the follicle was compared after different circulation times (2 min to 12 hrs). After a circulation time of two minutes the ferritin was found in the pericapillary region, in the intercellular spaces of the theca, and in the basal lamina of the follicle (Figures 25 and 27). The tracer could be found in the intercellular spaces between the granulosa cells of large follicles.
After a five minute circulation time, there is an increase in the ferritin concentration of the pericapillary region and in the follicle (Figure 28). The tracer is still absent from the interendothelial cell junction, and present in the plasmalemmal vesicles. Ferritin can now be seen in the membrane bound vesicles of cells near the capillaries [e.g. pericytes (Figure 29), thecal cells, smooth muscle cells]. The ferritin concentration is also higher in the intercellular spaces of the more mature follicles and is now visible in the zona pellucida. The intercellular spaces of the unilaminar follicles lack the tracer.

There is another increase in the general concentration of ferritin in the areas described above, after a circulation time of 15 minutes. The tracer can now be seen in membrane bound vesicles of the granulosa cells (Figure 30). The vesicles appear uniformly filled with the tracer. At this time a few ferritin molecules can be seen in the intercellular spaces between the follicle cells of the unilaminar follicle.

Figure 31 is representative of a capillary after a one hour circulation time. The tracer is still absent from the interendothelial cell junctions, and present in the plasmalemmal vesicles. The concentration
of ferritin is again higher throughout the follicular apparatus (Figure 32). More ferritin can be seen in the intercellular spaces between the follicle cells of the unilaminar follicle (Figure 33) along with evidence of uptake into the oocyte (Figure 34). The zona pellucida appear to contain a higher concentration of the tracer than is found in the antral fluid, indicating a possible accumulation of the tracer in this region.

The longer the circulation times (6 hrs to 12 hrs) the greater is the concentration of the tracer and greater is cellular uptake in the areas previously described.

**D. Ferritin Experiments on Sexually Mature Mice**

The mice were either in the metestrous or proestrous phase of the estrous cycle. They were injected (I.V.) with 50 mg ferritin in 0.2 ml saline. The tracer was allowed to circulate for either two or five minutes before decapitation.

The saline injected controls exhibited the same capillary morphology and the same general follicular morphology as was previously described.

1. **Proestrous Mice -- 2 Minute Circulation Time**

(Figure 35) Versus the Metestrous Mice -- 2 Minute Circulating Time (Figures 36 and 37)

The results of both experiments demonstrate the
same pattern of ferritin leaving the capillaries; e.g. an absence of the tracer in the interendothelial cell junctions, and the presence of the tracer in the plasmalemmal vesicles of the endothelium. The ferritin was found in both cases within the basal lamina surrounding the follicles. There appeared to be a higher concentration of ferritin in the follicles of the proestrous animals versus the metestrous animals. The capillary wall was the only region limiting the passage of the tracer to the follicles.

2. Proestrous Mice -- 5 Minute Circulation Time
(Figures 38 and 39) Versus Metestrous Mice -- 5 Minute Circulation Time (Figures 40 and 41)

The results of the two experiments demonstrated higher concentrations of ferritin in the perifollicular region when compared to the 2 minute circulation times. The manner of tracer passage across the capillary was the same. The concentration of ferritin still appeared in the follicles of the proestrous animals. Some tracer can now be found in the intercellular spaces of unilaminar follicles in the proestrous animals while none could be observed in the metestrous animals. No tracer could be found in the oocytes after this circulation time. The zona pellucida of the proestrous mice appeared to
accumulate more of the tracer than the zona of the metestrous animals.

E. Light Microscopic Observations

The following results were correlated with the ultrastructural observations presented above.

1. Controls and HRP Injected Animals (Figures 42-45)

The general histology of the control and tracer injected tissue is the same. The peroxidase reaction product can be seen in all the regions described in the ultrastructure studies. The controls confirm the absence of a positive peroxidase reaction product in the tissue.

2. Ferritin Injected Animals (Figures 46-50)

The appearance of ferritin, using Perl's staining method, is illustrated in Figure 46. After a circulation time of two minutes, the ferritin can be seen in the vasculature along with some being present in the intercellular spaces (Figures 47-49). The tracer can easily be seen in the intercellular spaces after a five minute circulation time. Figure 50 demonstrates the presence of ferritin in the granulosa layer and zona pellucida of the follicle.
F. Semiquantitative Results on Ferritin Leaving the Capillaries

The capillaries chosen for the measurements were found in the perifollicular region of follicles with beginning antrum formation in the immature mice, and the large follicles of the metestrous and proestrous mice.

The measurements of ferritin concentration were taken from random photomicrographs at a magnification which allows individual ferritin molecules to be resolved (representative electron micrograph, Figure 51, X64,000). It was also essential that enough surface area was available on the micrograph for molecule counts and surface area measurements to be taken in the capillary lumen and pericapillary region. This became a problem because most of the capillaries have red blood cells filling the lumen of the vessel. The surface area for each photograph was measured three times and the average used as the final value.

The percentage of ferritin to leave the capillary was calculated using the number of ferritin molecules per unit area in the lumen and the number of tracer molecules per unit area in the adjacent pericapillary region (out/in = % of ferritin to leave the capillary).

The results (Tables 7 and 8) indicate an increase
in the percentage of tracer found in the pericapillary region as the circulation time increases to five minutes. The percentage then lowers after the 15 minute and one hour circulation time. The results of the values from the proestrous and metestrous animals demonstrates a certain degree of variability. This will be discussed later in the paper.

G. Scanning Electron Microscopy

SEM was used in an attempt to study the vasculature of the perifollicular region with the expectation of receiving information which might complement the transmission electron microscopic data. Larger follicles were studied in cross section with the goal of viewing the vessels of the thecal layer (Figures 52 and 53). Because of the lack of magnification and resolution in the SEM, the ultrastructural detail of the capillary endothelium could not be studied.
<table>
<thead>
<tr>
<th>CIRCULATION TIME</th>
<th>REACTION PRODUCT IN CAPILLARY WALL</th>
<th>REACTION PRODUCT IN INTERCELLULAR SPACES OF THE FOLLICLE</th>
<th>REACTION PRODUCT IN GRANULOSA CELL VESICLES</th>
<th>REACTION PRODUCT IN OOCYTE VESICLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 sec</td>
<td>Throughout entire junction, few vesicles labelled</td>
<td>Entire, follicle of all types</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1 min</td>
<td>Same, with more vesicles labelled</td>
<td>Entire, moderate intensity in all follicles</td>
<td>Very few</td>
<td>None</td>
</tr>
<tr>
<td>2 min</td>
<td>Same, greater no. vesicles labelled</td>
<td>Same, with accumulation in zona and basal lamina</td>
<td>More, in periphery of cell</td>
<td>None</td>
</tr>
<tr>
<td>5 min</td>
<td>Same, most vesicles labelled</td>
<td>Same</td>
<td>Same</td>
<td>None</td>
</tr>
<tr>
<td>10 min</td>
<td>Same</td>
<td>Same, greater intensity</td>
<td>Same</td>
<td>Occasionally in oocytes of unilaminar follicles</td>
</tr>
<tr>
<td>15 min</td>
<td>Same</td>
<td>Same</td>
<td>Same, with some deeper in the cell</td>
<td>Same</td>
</tr>
<tr>
<td>30 min</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>More seen in oocyte of younger follicle &amp; rarely in larger follicles</td>
</tr>
</tbody>
</table>

*Extent of tracer penetration the same for sexually immature and sexually mature mice
<table>
<thead>
<tr>
<th>CIRCULATION TIME</th>
<th>FERRITIN IN CAPILLARY WALL</th>
<th>FERRITIN IN INTERCELLULAR SPACES OF FOLLICLE</th>
<th>FERRITIN IN GRANULOSA CELL VESICLES</th>
<th>FERRITIN IN OOCYTE VESICLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>Absent in junction, some vesicles labelled</td>
<td>Found in basal lamina, occasionally in between granulosa cells &amp; zona of larger follicle</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5 min</td>
<td>Same</td>
<td>Slightly greater conc., accumulating in zona</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>15 min</td>
<td>Same, with more vesicles labelled</td>
<td>Found in unilaminar follicles</td>
<td>Occasional</td>
<td>None</td>
</tr>
<tr>
<td>1 hr</td>
<td>Same</td>
<td>Entire, in all stages of follicle development</td>
<td>More vesicles labelled than above</td>
<td>Occasional particles in oocytes of unilaminar follicles</td>
</tr>
<tr>
<td>6 hrs</td>
<td>Same</td>
<td>Same, greater accumulation</td>
<td>Same, accumulation into larger vesicles</td>
<td>More in oocytes of unilaminar follicles rarely in oocytes of more developed follicles</td>
</tr>
<tr>
<td>12 hrs</td>
<td>No increase</td>
<td>No increase</td>
<td>No increase</td>
<td>No increase</td>
</tr>
</tbody>
</table>

*Extent of tracer penetration and cellular uptake was the same in the sexually immature and sexually mature animals*
TABLE 8

SEMIQUANTITATIVE MEASUREMENTS OF THE FERRITIN EXPERIMENTS**
(Percentage of ferritin to leave the capillary)

<table>
<thead>
<tr>
<th>IMMATURE ANIMALS</th>
<th>Circ. Time</th>
<th>2 min</th>
<th>5 min</th>
<th>15 min</th>
<th>1 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>10%</td>
<td>29.7%</td>
<td>28.7%</td>
<td>16.2%</td>
<td>37%</td>
</tr>
<tr>
<td>S.D.</td>
<td>4.5</td>
<td>8.2</td>
<td>15.5</td>
<td>5.1</td>
<td>4.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PROESTROUS ANIMALS</th>
<th>Circ. Time</th>
<th>2 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>16.7%</td>
<td>18.1%</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>12.2</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>METESTROUS ANIMALS</th>
<th>Circ. Time</th>
<th>2 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>5.1%</td>
<td>26.0%</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>3.7</td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates animal in which all measurements were taken from one size of follicle

**Random photomicrographs from 4 different mice for each circulation time
<table>
<thead>
<tr>
<th>Table 9</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>2 min.</th>
<th>5 min.</th>
<th>15 min.</th>
<th>1 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>M</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>M</td>
<td>I*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I*</td>
<td>I</td>
<td>M</td>
<td>M</td>
<td>I</td>
</tr>
</tbody>
</table>

Percentage of ferritin to leave the capillaries at different circulation times

- P - Proestrous Mice
- M - Metestrous Mice
- I* - Individual Immature Mice
- I - Immature Mice
V. DISCUSSION

A. The Morphology and Permeability of the Capillary Endothelium

The conclusions at a circulation and reproductive phenomena workshop emphasized the need for the type of investigation presented in this paper. It became apparent that there is a need for more investigation concerning the degree to which the vasculature regulates the function of the reproductive organs, and concerning the effect of hormones on the blood flow and vascular permeability (Moor, 1970). In addition, in a recent editorial, Greenwald (1972) expressed concern about the lack of recent studies on the vasculature of the ovary with relation to reproductive phenomena. The present study does not provide all the information asked for in the above statements, but does present base-line information regarding the morphology of the perifollicular capillary bed and the permeability characteristics of these vessels.

This section of the discussion deals with the fact that macromolecules found in the circulation must first leave the perifollicular capillary bed in order
to enter the ovarian follicle. Some of the work leading to the present study of the perifollicular capillaries will be discussed first.

Earlier investigations, using systemically injected trypan blue (Burr and Davies, 1951) and Evans blue (Zacchariae, 1958), reported an increase in the permeability of the rabbit ovarian follicles one and a half hours prior to their ovulation. The assumed reasons for this increase in permeability were: a reaction to hormone changes (Burr and Davies, 1951) and a reaction to a preovulatory enzymatic splitting of acid mucopolysaccharides in the follicular fluid causing an increase in intrafollicular colloid-osmotic pressure (Zacchariae, 1958).

In addition to studies on the passage of dyes from the systemic circulation into the follicle, later papers presented evidence that serum macromolecules could be identified in the follicle and oocyte (Glass, 1961; 1966; 1968; 1970; and Mancini, 1963). They showed that the macromolecules were not synthesized in the follicle, but that they originated from the systemic circulation.

This led some investigators to study the perifollicular capillary bed of the ovary. Ultrastructural
studies on the capillary bed of the corpus luteum showed the presence of gaps in the endothelium which were large enough to allow passage of ferritin and india ink into the pericapillary space (Morris and Sass, 1966). Such gaps were not observed in the capillary bed of other ovarian regions. However, similar gaps were found in the capillaries of the follicular apex just prior to ovulation (Byskov, 1969). Thus evidence was beginning to accumulate indicating that the entire ovarian capillary bed is not homogeneous in its morphology.

The present study has shown the morphology of the perifollicular capillary bed in sexually immature, and sexually mature proestrous and metestrous mice. This was carried even further by studying the perifollicular capillaries around follicles at different stages of development. The results demonstrate that the capillaries are made up of a continuous endothelium, lacking gaps or pores. The capillaries exhibited the type of endothelium similar to that described by Karnovsky (1969) and Bruns and Palade (1968) in skeletal muscle. Therefore, gap formation in the ovaries of the mouse must occur later in proestrus or early estrus just prior to ovulation.

The next step in this paper was to study the
passage of tracers across the perifollicular capillary endothe-lium. The tracers chosen were ferritin and horseradish peroxidase. They are different in molecular weight and diameter. HRP is a smaller molecule with a molecular weight of 40,000 (Keilin and Hartree, 1951) and a molecular diameter of 25-30 A (Karnovsky, 1967). Ferritin has a molecular weight of 500,000 and a molecular diameter of 110 A (Farrant, 1954; Harrison, 1959). In addition the tracers do not alter the permeability of capillaries in mice (Cotran and Karnovsky, 1966; Bruns and Palade, 1968).

The results indicated that the HRP left the capillary lumen rapidly (30 seconds). Most of the tracer passed through the interendothelial cell junctions and with some transport by the pinocytotic vesicles (Anderson, W. 1972) in the sexually immature rat ovary. He also found a rapid exit of the HRP from the perifollicular capillaries. But he did not study the sexually mature rat at the different phases of the estrous cycle.

The ferritin was slower in leaving the perifollicular capillaries. The junctions of the capillaries were impermeable to the larger tracer (ferritin). It was found in the vesicles of the endothelium; thus vesicular transport accounts for the exit of ferritin
from the capillaries. This was the case in the immature, proestrous, and metestrous mice. These results also are in agreement with the findings of Anderson (W. 1972) in the immature rat ovary.

The semiquantitative results on the percentage of ferritin found in the pericapillary spaces demonstrated high variability in the final values. A possible explanation could be that the capillary bed consists of two segments; arterial and venous (Rhodin, 1968; Hauck, 1969; Casley-Smith, 1971; Davis and Landau, 1966). A permeability gradient has been shown to exist between the two segments (Casley-Smith, 1971; Hauck, 1969). It should also be mentioned that studies of the above type were conducted on the mesentery capillary bed. This allows for controlled observation on the two segments (arterial and venous). The capillary bed around a follicle consists of a complex wreath of vessels, and it is not possible at the ultrastructural level to differentiate between arterial and venous capillaries. Thus no correlation could be made between the measurements taken and the segment of the capillary bed being studied at that time.

The present paper demonstrates two modes of molecular passage from the perifollicular capillary, depending on the size of the tracer being used; (1) interendothelial
cell junctions; and (2) the plasmalemmal vesicle.

The morphology of the interendothelial cell junction does vary with respect to the organ being studied. It may be a zonula occludentes (Bruns and Palade, 1968) or a macula occludentes (Karnovsky, 1969). It was important to determine the type of endothelial cell junction that exists in the capillaries of the mouse ovary, since variation in the capillary structure is so fundamental that one must caution against using measurements made on one capillary bed with respect to a capillary of a different type (Bennett et al., 1959). It was also important to compare the perifollicular capillaries observed in this paper with the findings on the capillary bed of the corpus luteum (Morris and Sass, 1966) and of the preovulatory follicle (Byskov, 1969).

The other factor to discuss is the effect of hormones on the endothelial cell junction of the microcirculation. Alteration in the serum levels of steroid hormones have been shown to affect the morphology of endothelial junctions inducing an increase in permeability of capillaries in the rat vagina (Carsten and Merker, 1965). But variation in capillary permeability to ferritin and HRP was not observed in the present paper.

Vesicular transit time across the endothelium
has been estimated to be as short as one second (Karnovsky and Shea, 1970) or as long as 3-5 seconds (Casley-Smith and Chin, 1971). The median vesicle attachment time to the luminal side or the tissue side of the endothelium has been estimated to be 2 to 3 seconds (Casley-Smith and Chin, 1971). The initial formation of the vesicle is energy dependent, but it has been shown that the population of vesicles free in the cytoplasm does not vary with changes in cellular activity (Casley-Smith, 1969b; Jennings and Florey, 1967). The number of vesicles found free in the cytoplasm of the endothelium did not appear to vary in the different experimental samples of the present study.

Vesicular transport appears to be the way in which ferritin leaves the capillary. Since none of the ferritin was found in the endothelial cell junctions, this would also explain why the ferritin was slower in leaving the perifollicular capillary.

Not all the vesicles were labelled with ferritin, regardless of the circulation time for the tracer. One explanation would be the phenomenon of molecular sieving, which can limit the number of macromolecules entering the open vesicle (Casley-Smith and Chin, 1971). The other would be the fact that the plane of section through
the vesicle may have missed the ferritin molecules contained within.

Another constituent of the capillary wall is the basal lamina surrounding the endothelium. The morphology of this extracellular layer was found to be the same as was described by other investigators (Bruns and Palade, 1968; Karnovsky, 1969; Clementi and Palade, 1969). In the present study it did not accumulate the tracers or stop their passage into the pericapillary region. This is in agreement with the findings of Bruns and Palade (1968), Clementi and Palade (1969), and Anderson (W. 1972).

The pericytes, sometimes found adjacent to the basal lamina of the capillary, did not restrict the passage of either tracer. There was eventual uptake of the tracers by the pericytes after longer circulation times.

B. The Morphology and Permeability of the Interstitial Spaces of the Perifollicular Region

After the tracers leave the perifollicular capillaries, they must pass through the intercellular spaces of the perifollicular region before reaching the basal lamina of the follicle. In this study the tracers passed rapidly through this region, regardless of the phase of
the estrous cycle of the experimental animal. This is in agreement with the conclusions of Wiederhielm (1968). He found that the free fluid compartment of the interstitial space is sufficient to allow free passage of macromolecules, even though the ground substance (e.g. hyaluronic acid and chondroitin sulfate) has a meshwork that will exclude macromolecular passage. Anderson (W. 1972) also found the interstitial spaces of the perifollicular region to be freely permeable to macromolecular tracers. In the present study, the cells of this region were found to take up the tracers after longer circulation times. Therefore the results of the present study demonstrate that the interstitial spaces of the perifollicular region do not represent any type of influential barrier to the permeability of the follicular apparatus.

C. The Morphology and Permeability of the Basal Lamina of the Ovarian Follicle

The basal lamina of the follicle was observed to be an amorphous layer of extracellular material. It was similar in electron density to the basal lamina surrounding the endothelial cell of the capillary. This layer varies in thickness with respect to the stage of follicular development, but the electron density did not vary during the phases of the estrous cycle. Some investigators
believe this layer may restrict the passage of large molecules. Anderson (W. 1972) observed the accumulation of ferritin against the basal lamina of the follicle irrespective of the stage of follicular development. He concluded that molecules of the size of ferritin cannot pass through the basal lamina of the immature rat follicle. This was not the case in the present study. Ferritin was found inside the basal lamina within minutes after its injection into mice.

The reason for the difference in the observation of the present study and that of Anderson (W. 1972) can only be speculated. First there may be a species difference in the permeability of the follicular basal lamina. Second, it may be a result of differences in the concentration of ferritin injected into the animals. But, Anderson (W. 1972) does not indicate that concentration of ferritin injected. Morris and Sass (1966), in their tracer experiments on the rat ovary, did not report whether the ferritin penetrated the basal lamina of the follicle. The present study used different doses of ferritin (90 mg - 30 mg) injected into sexually immature and sexually mature mice, and showed the basal lamina to be permeable to the tracer in all cases.

The rapid passage of HRP, a smaller tracer,
through the basal lamina was observed in the present study and by other investigators, e.g. Anderson (E. 1967) in the rabbit, and Anderson (W. 1972) in the immature rat.

D. The Permeability of the Granulosa Layer of the Follicle

In the unilaminar follicles, the association between the follicle cells themselves and the oocyte appears to be one of close morphological apposition and close functional relationship (Weakley, 1966; 1967; Odor and Blandau, 1969; Franchi and Mandl, 1962; Chiquoine, 1960). This may be an influential factor in expediting the passage of macromolecules from the systemic circulation to the oocyte of this type of follicle.

The present study showed that HRP can pass freely through the intercellular spaces between the granulosa cells of the unilaminar follicle. Some uptake of thetracer by the granulosa cells is observed, but it does not appear to be a means of intracellular transport of the tracer to the region of the oocyte. The tracer was found in the intercellular spaces of the granulosa cells within 30 seconds after injection. The results are in agreement with the findings of Anderson (E. 1967) on the rabbit, and Anderson (W. 1972) on the sexually immature rat.
The situation is not the same when referring to the passage of a larger tracer such as ferritin. It was found that ferritin did not pass easily through the intercellular spaces between the granulosa cells of unilaminar follicles. The ferritin was found in this region only when the circulation time was at least 15 minutes. The tracer was not observed in the membrane bound vesicles of the oocyte until an hour after injection of the ferritin. This suggests that the ferritin may have been retarded in its passage to the oocyte because of the close apposition of the follicle cells.

On the other hand, occasional ferritin molecules could be found within two minutes of injection in follicles with beginning antrum formation up to and including the proestrous follicle. In these types of follicles the granulosa cells are not as closely apposed at the site of the basal lamina. Thus it appears that due to the morphological arrangement of the granulosa cells, that the more mature follicles are more permeable to a tracer the size of ferritin than the unilaminar follicles.

The ferritin is also found eventually in membrane bound vesicles of the granulosa cells, but it does not appear to be a mechanism for transporting the molecules to the oocyte.
There are not other papers describing the passage of ferritin in the intercellular spaces of the granulosa layer. Thus the observations reported in the present paper cannot be compared. Anderson (W. 1972) found that the basal lamina surrounding the follicles of the immature rat ovary did not allow the passage of ferritin into the granulosa layer. The only other explanation for Anderson not finding ferritin in the follicle could be the difficulty in being able to observe low concentrations of ferritin molecules. The electron dense core of ferritin is only 55 A in diameter. In the present study bismuth subnitrate (Ainsworth and Karnovsky, 1972) was used to enhance the electron opacity of the ferritin molecule. This makes it easier to observe the tracer under the electron microscope.

Controls (saline injection alone) were conducted in order to insure that one was not looking at electron dense particulate matter and interpreting it to be ferritin molecules.

Anderson (W. 1972) does indicate that preliminary results on additional studies on sexually mature rats show that the preovulatory follicles found in ovaries with corpora lutea present are permeable to the ferritin. But the present study demonstrates that all follicles in
the sexually immature mice (no corpora lutea) and sexually mature mice in proestrus and metestrus are permeable to the tracer.

Therefore the results of the present tracer studies demonstrate that the tracers do enter the granulosa layer. This macromolecular passage to the oocyte is not by intracellular transport, but is a passive diffusion through the intercellular spaces.

E. The Morphology and Permeability of the Zona Pellucida

Findings of the present paper demonstrate that the zona pellucida is freely permeable to both ferritin and HRP molecules.

Austin and Lovelock (1958) have also found that alcian blue (approx. mol. wt. 12,000) and digitonin (approx. mol. wt. 750) were able to penetrate the zona pellucida. But they could not observe heparin (approx. mol. wt. 16,000) reaching the oocyte. Therefore they concluded that the zona pellucida was an effective barrier to molecules in the weight range of heparin. In contrast, it has been shown the large serum proteins (mol. wt. 55,000 and higher) can pass through the zona pellucida of the mouse (Glass, 1961, 1963). Ferritin and HRP can penetrate the zona pellucida of the rat,
mouse and rabbit blasocyst (Hastings et al., 1972). The zona pellucida of the rabbit (Anderson, E. 1967), the rat (Anderson, W. 1972) and the mouse (present study) oocyte are freely permeable to HRP. The only evidence that the zona pellucida of the follicular oocyte is permeable to ferritin is demonstrated in the present study.

The concentration of tracers in the zona pellucida appears to be higher than in the adjacent follicular fluid. Either the higher density of the zona or a greater binding at fixation might account for the difference in distribution.

It still remains possible that the zona pellucida may be a barrier to molecular aggregates larger than individual ferritin molecules.

The zona pellucida is generally considered to be made up of glycoprotein since it gives a positive reaction for polysaccharides and is soluble in proteolytic enzymes (Soupart and Noyes, 1964). The explanation for the different results on the permeability of the zona is not complete. For example, it is possible that charge effects might influence penetration of specific molecules through the zona, but at present there is no definite proof that this is the case. The only other possibility is that there is a species difference in the
chemical nature of the zona which in turn could affect its permeability, but again this has not been determined.

F. Oocyte Uptake of Macromolecules

Another goal of the present paper and other related investigations was to determine whether the oocyte will incorporate macromolecules originating from the systemic circulation. The present study was interested in comparing the uptake of ferritin and HRP into follicular oocytes.

There was evidence of cellular uptake of HRP into the oocytes of the unilaminar follicles. But the oocytes of the more mature follicles rarely demonstrated HRP uptake. Anderson (W. 1972) observed a similar pattern of cellular uptake by the oocytes of the sexually immature rat. However, Anderson (E. 1967) found a greater uptake into oocytes of larger follicles, while little could be found in the oocytes of the unilaminar follicle.

In the present paper, HRP was found in vesicles open to the extracellular space adjacent to the oolemma. It was also found in membrane bound vesicles located in various regions of the oocyte cytoplasm. Thus it may be assumed that the tracer is being incorporated into the oocyte by pinocytosis. This is in agreement with the findings of Anderson (E. 1972). He further postulated
about the possible fate of the exogenously administered proteins in the cells of the follicle. He believed the proteins were taken into the cell by pinocytosis; then the membrane bound vesicles containing the tracer became fused with a primary lysosome derived from the Golgi body, where digestion takes place. The fate of the residual body of digested material is not known. It may be removed from the cell by exocytosis.

Another question arises with regard to reasons why selective uptake by the oocyte depends on the state of maturation. There have been investigations which shown evidence that there is a relationship between changes in the glycocalyx (cell coat) and the overall charge of the molecule being incorporated into the cell (e.g. Schumaker, 1958; Fawcett, 1965). After the glycocalyx and the macromolecules are bound, a coated vesicle containing the molecules is formed. This change in the glycocalyx is believed to occur in localized areas on the plasmalemma of the cell. Therefore it may be possible that the overall charge of the tracer molecule and the binding characteristics of the glycocalyx may influence whether the macromolecule will be incorporated into the cell. These factors may also be characteristic of the oolemma and the factors may be changing as the oocyte
matures, thus changing the selectivity of the oocyte plasmalemma. The above is speculation with respect to the oocyte and has not been verified.

The present study showed a greater uptake of ferritin in the less mature oocytes. This was similar to the results of the HRP experiments. The only other investigations utilizing ferritin as a tracer to study follicular permeability were conducted by Morris and Sass (1966) and Anderson (W. 1972). Neither of the papers noted ferritin penetration through the basal lamina of the follicle. Therefore no comparison can be made on the uptake of ferritin by the oocyte.

The result of the studies mentioned above, along with the present paper, partially agree and partially disagree with the light microscopic evidence concerning follicular permeability. For example, it has been shown that: systemically injected proteins are found in the cytoplasm of follicular oocytes (Glass, 1961; Mancini et al., 1963); the transfer of macromolecules into the follicle is stage dependent (Glass and Cons, 1968); and such a transfer may be selective as to molecular species (Glass, 1966). It is evident that the agreement lies in the fact that macromolecules from the systemic circulation can be found in the ovarian follicle and its oocyte.
Also the present study demonstrates that the uptake of the molecules into the oocyte is dependent on the maturity of the oocyte. The disagreement is in the specificity of the oocyte to different molecular species. In the present paper both tracers were taken up by the oocyte regardless of differences in molecular diameter and weight. On the other hand, Glass found that bovine albumin (approx. mol. wt. 69,000) entered the oocyte while bovine globulin (approx. mol. wt. 150,000) did not.

G. Dynamics of the Ovarian Vasculature

Earlier investigators observed changes occurring in the general vascular pattern of the ovary during growth, and the phases of the reproductive cycle; e.g. Bassett (1943) in the rat ovary; Delson et al. (1949) in the human ovary; and Davies (1951) in the rabbit ovary. Results of the present study demonstrate that the ultrastructural morphology of the perifollicular capillary bed does not change when comparing it in the sexually immature mice or with mice in the proestrus and metestrus phases of the estrous cycle.

Some investigators have shown ultrastructural differences in the morphology of the ovarian capillaries. For example, gaps have been found in the endothelium of the corpus luteum (Morris and Sass, 1966)
and in the capillaries of the follicular apex just prior to ovulation (Byskov, 1969). These papers and the present study complement each other, producing a complete classification of the types of capillary bed found in the ovary. The capillary endothelium remains continuous in its morphology until a time point just before ovulation. It is then that the capillaries near the apex of the pre-ovulatory follicle develop gaps in the endothelium. Finally the gaps become more prevalent in the capillaries of the corpus luteum. Thus there is a progressive change occurring in capillary morphology during follicular maturation, ovulation and corpus luteum formation.

H. Significance of Heterosynthetic Macromolecular Transport to the Oocyte

Schechtman (1955) refers to macromolecules, which leave the systemic circulation and are taken up by the cells outside the vasculature, as heterosynthetic molecules.

The present study demonstrated the passage of macromolecular tracers from the perifollicular capillary bed into the follicles and the cells it contains. Now that the passage of macromolecules has been described and compared with similar investigations, the significance of this macromolecular passage should be
discussed.

It has been suggested that the heterosynthetic molecules incorporated into the oocyte may serve as a mechanism for internalizing nutritive substances for the oocyte (Anderson and Beams, 1960). The incorporation of serum proteins into the oocyte has been shown by Glass (1961) and Mancini et al. (1963). Glass (1966) also showed that some of the macromolecules found in the oocyte are identical to macromolecules found in the plasma. Therefore the possibility does exist that the heterosynthetic macromolecules are a nutritive source of necessary compounds not synthesized by the oocyte itself.

On the other hand, there may be other reasons for the incorporation of macromolecules by the oocyte. Albumin has been described as a carrier for other molecules (Foster, 1960). With this in mind other speculations for the role of serum proteins have been presented: (1) the albumin may be carrying some type of molecule needed by the oocyte; (2) the affinity of albumin for ions, thus serving as a non-specific covering, may prevent the premature utilization of particular substances being stored in the cytoplasm of the oocyte (Glass, 1971). Thus just using the example of albumin, it can be appreciated that the heterosynthetic macromolecules
could significantly influence the normal development of the oocyte. The results of the present study show that foreign macromolecules are capable of penetrating the follicle and entering the cells it contains. This indicates the possibility of other foreign macromolecules being able to enter the oocyte and affecting the development of this cell. However this remains to be further studied.

I. The Effects of Hormones on the Permeability of the Follicular Apparatus

Since it has been shown that changes in serum hormone levels can affect the morphology of the ovarian follicles and the corpus luteum (Allen, 1922; Long and Evans, 1922; Boling et al., 1941; Mandl and Zuckerman, 1952; Jones and Krohn, 1961; Bingel and Schwartz, 1969), it is now important to discuss hormonal effects on the permeability of the follicular apparatus.

Evidence that hormones can influence the permeability characteristics of the ovarian vasculature was presented in the review of this paper (Wurtman, 1964; Szego, 1965; Lipner, 1971; Wolff et al., 1967; Piascek and Huth, 1971; Forbes and Glassen, 1972). This could influence the availability of plasma macromolecules to the components of the follicular apparatus.
The present paper was involved in determining whether the perifollicular capillary bed was being influenced by variations in serum hormone levels. No exogenous hormones were injected into the animals. The normal changing levels of endogenous hormones during the estrous cycle were used for this study.

Although knowledge of the estrous cycle of the mouse dates back to the classic study by Allen (1922), information concerning the levels of serum hormones during the estrous cycle were derived from recent investigations using more sensitive assay techniques. The results from the papers will demonstrate that the serum hormone levels are significantly different in the proestrous and metestrous mice used in the present study.

Bingel and Schwartz (1969) conducted a series of studies on the cyclic mouse. They found that the pituitary LH content did not significantly vary with the cycle, but that the mean potency appeared highest on the first day of diestrum. Their results from barbital treated mice indicated that there was a late afternoon to early evening critical period during proestrus (14:00-16:00-17:00 hours) for LH release in the unmated cyclic mouse (L14:D10 light cycle). This held true for both the 4 or 5 day cyclic mouse. Kovacic and Parlow
(1972) studied the serum levels of FSH and LH in the mouse, using the radioimmunoassay system of NIAMD. The results demonstrated a large surge of serum LH on the late afternoon and early evening of proestrus (17:00 to 18:30 hours); an elevation of serum FSH on the early morning of estrus (between 7:30 and 11:00 hours). In addition, the levels of serum LH and FSH were significantly lower on the day of metestrus when compared to the levels on the evening of proestrus.

Murr et al. (1973) also demonstrated differences in plasma levels of FSH and LH during the estrous cycle of the mouse. They found the mean peak of LH to be at 17:00 hours on the day of proestrus, and it decreased to basal level by the morning of estrus. The level of FSH reached a peak during proestrus, but the peak was not as sharp as was found for LH.

Thus it can be assumed that the serum LH levels and FSH levels significantly change during the estrous cycle, especially when comparing the serum levels on the evening of proestrus and the evening of metestrus.

A cyclic variation was also shown for the steroid hormone levels during the estrous cycle in the rat. Estrogen reached its highest levels on the afternoon of proestrus (Schwartz, 1969). Hashimoto et al. (1968)
measured the progesterone levels in the ovarian vein, and found that the highest level was reached on the afternoon of proestrus.

With the above information in mind, it was decided that in order to observe any variation in the microvasculature and morphology of the follicular apparatus due to changes in the levels of endogenous hormones, mice should be chosen which are in the following reproductive states: (1) the sexually immature mouse just prior to the onset of reproductive cyclicity; (2) mice in proestrus (between 17:00 and 18:00 hours); and (3) mice in metestrus (between 17:00 and 18:00 hours).

The results of the present study show that the morphology of the perifollicular capillaries remained the same in each of the above experimentals. This also held true when comparing the perifollicular capillaries surrounding follicles at different stages of development. All the capillaries possessed a continuous endothelium.

Semiquantitative measurements on the permeability of the perifollicular capillaries to HRP were not possible. This is because the tracer rapidly leaves the capillaries, and the reaction product for peroxidase cannot be measured accurately. It was possible to measure
the amount of ferritin leaving the capillaries because: the tracer leaves the capillaries at a slower rate, and the individual molecules can be counted (55 A ferric hydroxide core).

It was hoped that if there was any change in the permeability of the perifollicular capillaries to ferritin, it would be demonstrated in the results of the measurements. But the results showed a significant degree of variation in the final values.

One of the reasons for the variation might be attributed to the permeability differences between the two segments of the capillary bed. And, as it was mentioned earlier, it is impossible to differentiate the two segments at the ultrastructural level. The other possibility is that there is occasional leakage of ferritin through the venules. There were no examples of this type of leakage in the ultra-thin sections studied. Because one is viewing sections that are in the size range of 60-90 millimicrons, the possibility cannot be ruled out that the occasional leakage was missed. Therefore light microscopic studies, using Perl's stain for ferritin, were conducted in order to determine whether there was any abnormal leakage of the tracer from the vasculature. The results did not show any evidence of this type
of vascular leakage.
VI. SUMMARY AND CONCLUSIONS

The permeability of the follicular apparatus in the mouse ovary was studied at the ultrastructural level utilizing protein tracers (ferritin and horseradish peroxidase). Light microscopic studies were conducted for morphology, orientation, and determination of vascular leakage due to the tracers. The electron microscope was used to precisely localize the tracers and to determine the extent of tracer penetration after different circulation times. Semiquantitative measurements were made in an attempt to determine any variation in capillary permeability that may occur in the different experiments. The results of the paper are evaluated with respect to: variation in permeability that may be related to the molecular size and weight of the tracers; possible sites regulating permeability in the follicular apparatus; differences in cellular uptake of the tracers; and possible correlations between the permeability of the follicular apparatus with the changing levels of endogenous hormones during the estrous cycle of the mouse. The results achieved in this paper can be summarized as follows:
1. The dose of tracers along with the volume of physiological saline injected did not produce observable alterations in capillary permeability or in the morphology of the capillaries and venules. The quantity of tracer used in this study was below the dosage used by previous investigators where even their higher doses did not produce any changes in capillary permeability.

2. Light microscopic and electron microscopic observations demonstrated a rapid passage of HRP from the systemic circulation into the follicles (30 seconds). However, the situation was different with ferritin, since it was not observed outside the capillaries until two minutes following injection.

3. Ultrastructural observations on the perifollicular capillaries revealed the lining to be of continuous endothelium in immature, proestrous, and metestrous mice. The endothelium did not exhibit fenestrations or gaps. Nor did the morphology of the perifollicular capillaries change with respect to the different phases of follicular development.

4. Comparison of controls with the experimental animals showed that the morphology of the capillary endothelium was not changed by the tracers. In addition the general morphology of the follicular apparatus appeared
normal.

5. The rate and method by which the tracers left the capillaries differed. HRP left the capillaries rapidly, primarily through the interendothelial cell junctions and within pinocytotic vesicles to a minute degree. Ferritin, on the other hand, left the capillaries at a slower rate. Endothelial cell junctions were impermeable to the larger tracer. Instead, vesicular transport accounted for the transfer of ferritin out of the capillary. The only other site slowing the passage of ferritin was the intercellular spaces between the follicle cells of the unilaminar follicle. This was probably due to the close apposition of the cells.

6. As the circulation time for the tracer was lengthened, there was a proportional increase in the concentration of ferritin in the pericapillary region.

7. This study clearly demonstrates that the basal lamina of the follicle is permeable to both tracers.

8. The intercellular spaces between the granulosa cells are the primary pathways for the tracers to reach the oocyte. The ferritin and HRP freely diffuse through the intercellular spaces. There was some cellular uptake of the tracers by the granulosa cells, but it does not play an important role in tracer transport to the oocyte.
9. The zona pellucida was permeable to both of the tracers irrespective of whether the zona was in the process of development or already developed.

10. HRP was often found in the oocytes of follicles that were in the earlier stages of maturation, while little was present in oocytes of more mature follicles. Ferritin uptake was greater in the oocytes of unilaminar follicles, while the more mature oocytes rarely exhibited the presence of the tracer.

11. The semiquantitative results of ferritin passage out of the capillaries on random samples, indicate significant variation in the individual measurements. This may be the result of the permeability gradient that exists between the arterial and venous ends of the capillary bed. But, the recognition of any morphological differences between the two segments of the capillary bed is not possible at the ultrastructural level. The other possibility is venular leakage of the tracer. However, no leakage was observed with the concentration used in this study.

12. An attempt was made to correlate the permeability of the perifollicular capillary bed with the following factors: (1) the elevated FSH and LH serum levels during proestrus and the lower levels at metestrus; (2) the
elevated secretion levels of estrogen and progesterone during proestrus; (3) the known effects of hormones on ovarian blood flow and vascular permeability; and (4) the cyclic changes in ovarian morphology correlated with the phases of the estrous cycle. But no morphological differences were observed in the perifollicular capillary bed regardless of whether the animals were sexually immature or sexually mature (proestrous and metestrous phase of the estrous cycle).

In conclusion this study has demonstrated the extent of permeability in the follicular apparatus of the mouse ovary. The utilization of two protein tracers having different molecular diameters and molecular weights (similar size range to some serum proteins) has enabled the investigator to follow the passage of the tracers from the lumen of the perifollicular capillaries into the follicle, and their incorporation by the granulosa cells and the oocyte.

The intent of this study was to obtain base-line information on the morphology and permeability characteristics of the follicular apparatus, with emphasis on the perifollicular capillary bed. Future investigations will be concerned with the effects of exogenous hormones on the perifollicular microvasculature of the ovary.


Plate I

Figure 1. Simplified representation of a unilaminar follicle. It will be used to indicate the area sectioned in the electron micrographs. O: oocyte; g: granulosa layer; B: basal lamina of the follicle; C: capillary; I: interstitial tissue surrounding follicle.

Figure 2. Simplified representation of a secondary follicle. It will be used to indicate the area sectioned in the electron micrographs. O: oocyte; Z: zona pellucida; g: granulosa layer; B: basal lamina; T: thecal layer; C: capillary.
Plate II

Figure 3. Light micrograph of the mouse ovary showing the follicles (arrows) at different stages of development. O: oocyte; G: granulosa layer; T: thecal layer. Hematoxylin and eosin, X200.
Figure 4. Electron micrograph showing the relation of a perifollicular capillary (C) in the thecal layer (T) with the basal lamina (B) of the follicle. G: granulosa cell; R: red blood cell. Uranyl acetate, X5,600.
Plate IV

Figure 5. Electron micrograph of a capillary which is a few cell layers from the basal lamina (B) of the follicle. Note the type of junctions (J) in the capillary endothelium (E). T: thecal layer; G: granulosa cell; b: basal lamina of the capillary. Uranyl acetate, X16,000.
Plate V

Figure 6. Electron micrograph of a capillary next to the basal lamina (B) of the follicle. Note the plasmalemmal vesicles (arrows) in the endothelium (E). G: granulosa cell; R: red blood cell. Uranyl acetate, X32,000.
Plate VI

Figure 7. Electron micrograph showing the morphology of the basal lamina (B) of the follicle. Note the space (arrows) between the granulosa cells (G) at the site of the basal lamina. T: thecal layer. Uranyl acetate, X32,000.
Figure 8. Electron micrograph of a developing zona pel­lucida (Z). Note the difference in electron density between the zona and the follicular fluid (F). O: oocyte; G: granulosa cell. Uranyl acetate, X22,400.
Figure 9. A unilaminar follicle from an animal not injected with HRP, but incubated in a complete medium. Note the lack of any endogenous electron dense peroxidase reaction product. O: oocyte; G: granulosa cell; B: basal lamina. Uranyl acetate, X16,000.
Figure 10. Perifollicular region from an animal not injected with HRP, but incubated in a complete medium. Note the lack of any peroxidase positive activity. The red blood cell (R) reacts with the incubation medium causing a strongly electron dense appearance. Also observe that the capillary morphology is not altered by the incubation process. G: granulosa cell; E: endothelium; J: endothelial junction. Uranyl acetate, X32,000.
Figure 11. The distribution of the uniform electron dense HRP reaction product (arrows) after a 30 second circulation time. O: oocyte; g: granulosa cell; e: ovarian epithelium. Uranyl acetate, X8,000.
Figure 12. Endothelial cell junction showing the presence of the HRP reaction product along its entire length (arrows). Two minute tracer circulation time. R: red blood cell; E: endothelium. Unstained, X44,000.
Figure 13. Portion of the capillary endothelium (E) showing plasmalemmal vesicles containing the electron dense HRP reaction product (arrows). Two minute tracer circulation time. R: red blood cell. Unstained, X64,000.
Plate XIII

Figure 14. Example of the HRP reaction product (arrow) in a membrane bound vesicle of a granulosa cell (g). Five minute tracer circulation time. A: antrum. Uranyl acetate, X44,000.
Figure 15. Note the presence of the HRP reaction product in a forming pinocytotic vesicle (P). Also observe the reaction product in membrane bound vesicles (arrow) in the cytoplasm of the oocyte (O) and granulosa cell (g). Ten minute tracer circulation time. Unstained, X64,000.
Figure 16. Unilaminar follicle showing intensity of the HRP reaction product (arrows) after a 30 minute tracer circulation time. Note the cellular uptake by the granulosa cells (g). O: oocyte. Unstained, X22,400.
Plate XVI

Figure 17. General distribution and intensity of the HRP reaction product (arrows). Thirty minute circulation time. O: oocyte; g: granulosa cells. Uranyl acetate, X5,600.
Figure 18. Distribution of the HRP reaction product (arrows) in a follicle with beginning antrum formation. Note the accumulation of the electron dense reaction product in the zona pellucida (Z). B: basal lamina; g: granulosa cell; O: oocyte. Uranyl acetate, X5,600.
Figure 19. An example of abnormal leakage (arrow) of the tracer from the vessel due to the high concentration of ferritin injected (90 mg/5 ml into the animal). O: oocyte; g: granulosa cell. Bismuth subnitrate, X32,000.
Plate XIX

Figure 20. Electron micrograph of the capillary from a control, non-injected animal. Note the lack of any electron dense particles that could be interpreted as ferritin. Arrow: intercellular junction; E: endothelium; R: red blood cell; L: lumen; P: pericapillary region. Unstained, X64,000.
Plate XX

Figure 21. Electron micrograph of the zona pellucida (Z) in a control, non-injected animal. Note the lack of any ferritin like particles in the zona. O: oocyte. Unstained, X64,000.
Figure 22. Capillary (C) in the thecal layer of a follicle. The circulation time for the tracer was five minutes. Note that it is difficult to observe the presence of the tracer at this magnification. P: pericyte; T: thecal cell; G: granulosa cell. Bismuth subnitrate, X8,000.
Figure 23. Blunt intercellular junction of a capillary in a ferritin injected animal. Circulation time was five minutes. Note the lack of ferritin in the junction (arrow). L: capillary lumen; E: endothelium. Bismuth subnitrate, X70,000.

Figure 24. Interdigitating intercellular junction of a capillary from a ferritin injected mouse. Tracer circulated for five minutes. Note the absence of the tracer in the junction (arrow). L: capillary lumen; E: endothelium; P: pericapillary space. Bismuth subnitrate, X64,000.
Figure 25. Ferritin can be seen in the pericapillary region (P) and the basal lamina (B) of the follicle after a two minute circulation time. L: capillary lumen; T: thecal cell; g: granulosa cell. Bismuth subnitrate, X24,000.
Plate XXIV

Figure 26. Electron micrograph of the pericapillary endothelium (E) in a ferritin injected mouse, two minute circulation time. Note the ferritin labelled plasmalemmal vesicle (V) characteristic of the type observed in this study. L: lumen; P: pericapillary space. Bismuth subnitrate, X64,000.
Plate XXV

Figure 27. Electron micrograph of a follicle with beginning antrum formation. Ferritin circulation time of two minutes. Note the occasional ferritin molecules (arrows) in the intercellular spaces between the granulosa cells (G). B: basal lamina. Bismuth subnitrate, X64,000.
Figure 27. Electron micrograph of a follicle with beginning antrum formation. Ferritin circulation time of two minutes. Note the occasional ferritin molecules (arrow) in the intercellular spaces between the granulosa cells (G). B: basal lamina. Bismuth subnitrate, X64,000.
Plate XXVII

Figure 29. Section from a ferritin injected mouse. Five minute tracer circulation time. Note the uptake of the tracer (arrows) in the pericyte (P) of the perifollicular region. R: red blood cell; E: endothelium; L: capillary lumen. Bismuth subnitrate, X64,000.

Figure 30. An example of the ferritin in a membrane bound vesicle (arrows) of a granulosa cell (g). Fifteen minute tracer circulation time. B: basal lamina. Bismuth subnitrate, X64,000.
Figure 31. An electron micrograph of a capillary near the basal lamina of a follicle. Note that the concentration of ferritin in the capillary lumen is still high. Also observe the increased concentration of the ferritin in the intercellular spaces. E: endothelium; L: capillary lumen; B: basal lamina of the follicle; G: granulosa cell. Bismuth subnitrate, X22,400.
Figure 32. Observe the ferritin in the follicular fluid of the follicle. One hour tracer circulation time. Note the membrane bound vesicle containing a few ferritin molecules (arrow). G: granulosa cell; A: antrum. Unstained, X64,000.
Figure 33. An electron micrograph of a unilaminar follicle. Ferritin injection with a circulation time of one hour. Note the ferritin (arrows) in the intercellular spaces between the granulosa cells (G). Also observe the ferritin in the membrane bound vesicle (V) of the granulosa cell. B: basal lamina. Bismuth subnitrate, X64,000.
Figure 34. A section through the oocyte-granulosa cell interface. The injected ferritin circulated for one hour. Note the presence of ferritin (arrow) in between the oocyte (O) and granulosa cell (G). Ferritin can also be seen in membrane bound vesicles (V) of the oocyte. Unstained, X64,000.
Plate XXXII

Figure 35. Electron micrograph of the capillary endothelium. The section is from a proestrus mouse. Two minute tracer circulation time. Note the absence of the tracer in the endothelial cell junction (arrows). L: capillary lumen; E: endothelium. Bismuth subnitrate, X64,000.
Plate XXXIII

Figure 36. Perifollicular capillary from a metestrus animal. The ferritin circulated for two minutes. L: capillary lumen; B: basal lamina of the follicle; T: thecal cell; G: granulosa cell. Bismuth subnitrate, X32,000.
Figure 37. Intercellular space between granulosa cell of a follicle with a well developed antrum. Metestrus mouse. Ferritin circulated for two minutes. Note the occasional ferritin molecules (arrows) found in the intercellular space. G: granulosa cell; A: intercellular space. Bismuth subnitrate, X64,000.

Plate XXXIV
Plate XXXV

Figure 38. Zona pellucida (Z) from a proestrus mouse. Ferritin circulated for five minutes. Note the presence of the ferritin in the zona. O: oocyte. Unstained, X64,000.
Figure 39. Electron micrograph of a perifollicular capillary from a proestrus mouse. Ferritin circulated for five minutes. Note the pinocytotic vesicle (V) forming along with its ferritin content. E: endothelium; R: red blood cell. Unstained, X64,000.

Figure 40. Endothelium of a perifollicular capillary from a metestrus animal. Ferritin circulated for five minutes. Observe the lack of ferritin in the endothelial cell junction (arrows). E: endothelium; L: capillary lumen. Bismuth subnitrate, X64,000.
Figure 41. Oocyte (O) and its surrounding zona pellucida (Z) from a metestrus mouse. Note the presence of ferritin in the zona. Ferritin circulated for five minutes. Bismuth subnitrate, X64,000.
Plate XXXVIII

Figure 42. Light micrograph of a region between two follicles. HRP was injected, but the incubation medium was lacking DAB. Observe the normal morphology of the follicle and associated tissue. O: oocyte; g: granulosa cell; T: thecal layer. Toluidine blue, X1,000.

Figure 43. Light micrograph of an ovarian follicle. The animal was not injected with HRP, but was incubated in a complete medium. Note the absence of any endogenous peroxidase activity in the follicular apparatus. O: oocyte; Z: zona pellucida; g: granulosa layer; T: thecal layer. Toluidine blue, X800.
Plate XXXIX

Figure 44. Light micrograph of a follicle from an immature mouse. Note the presence of the HRP reaction product throughout the intercellular spaces of the tissue. HRP circulated for thirty seconds. B: basal lamina; g: granulosa layer; Z: zona pellucida; O: oocyte. Toluidine blue, X1,000.

Figure 45. Light micrograph of a follicle from an immature mouse. HRP circulated for two minutes. Note the intense HRP reaction product (arrows) throughout the follicle. O: oocyte; G: granulosa cell; B: basal lamina. Toluidine blue, X1,300.
Plate XL

Figure 46. Light micrograph of a 40 micron section through the mouse oviduct. The ferritin circulated for two minutes. Note the presence of the ferritin in the vessels of the oviduct (arrows). Perl's stain, no counterstain, X100.

Figure 47. Light micrograph of follicles (F) in the ovarian cortex. Ferritin circulated for two minutes. Note the localization of the ferritin in the vessels (arrows). Perl's stain, no counterstain, X200.
Plate XLI

Figure 48. Region of the mouse ovary. Note the presence of the blue stain for ferritin in the vessels (arrows), but lacking in the lymphatic vessels (L). Ferritin circulated for two minutes. O: oocyte. Perl's stain, nuclear red counterstain, X800.

Figure 49. Observe at higher magnification, that the blue stain for ferritin can be seen in the perifollicular regions (arrows). Ferritin circulated for two minutes. F: follicle. Perl's stain, no counterstain, X800.
Figure 50. Light micrograph of a mouse ovarian follicle. The ferritin circulated for one hour. Note the blue stain for ferritin in the thecal layer (T), the granulosa layer (G), and in the zona pellucida (Z). O: oocyte. Perl's stain, no counterstain, X600.
Plate XLIII

Figure 51. Electron micrograph of the capillary endothelium (E). Area enclosed in black lines represents the regions where the ferritin molecules were counted. R: red blood cell; L: capillary lumen; P: pericapillary space. Bismuth subnitrate, X64,000.
Figure 52. Scanning electron micrograph of a Graafian follicle. The arrows indicate the boundary of the follicle and point to the follicular antrum. Note the antral surface of the granulosa cells (g). The thecal layer (T) was scanned at higher magnifications in order to locate capillaries. X400.

Figure 53. Scanning electron micrograph of a blood vessel. The vessel is outlined with arrows. R: red blood cell. X2,500.
The dissertation submitted by Andrew F. Payer has been read and approved by members of the faculty of the Graduate School of Loyola University of Chicago.

The final copies have been examined by the chairman of the thesis committee and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

08/08/73

Date

Signature of Advisor