To the Graduate Council:

I am submitting herewith a dissertation written by Eugene Alfred Nau entitled "Gonadotropin and LDL Receptors of the Ovarian Granulosa Lutein Cell of the Pig." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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Vice Provost
and Dean of The Graduate School

GONADOTROPIN AND LDL RECEPTORS OF THE OVARIAN GRANULOSA LUTEIN CELL OF THE PIG

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Eugene Alfred Nau

June 1987

ACKNOWLEDGEMENTS

I would like to thank the members of my dissertation committee for their help; first and foremost Dr. John H. Abel for his encouragement, patience, and guidance in directing my life, research, and in the writing of this dissertation; Dr. John R. Kennedy for his encouragement and for assuming chairmanship of my committee in Dr. Abel's absence; Dr. Leaf Huang, Dr. John Koontz, and Dr. Roland Bagby for their valuable expertise in assisting me with my research and for their constructive criticism.

Appreciation is also expressed for Dr. Canfield, Columbia University, for his generous gift in supplying my research with human chorionic gonadotropin for radioiodination.

Recognition must also be given to the following undergraduates for their generous labor: Larry Love, Jennifer Hammer, Joe Kay, Bonnie Stopa, Chris Davenport, Eddie Moran, Eric Schaffer, John Davis, Marlene Brogan, and Patrice Sommers.

Last but certainly not least, I am extremely grateful to Maharishi Mahesh Yogi and his tradition of yogic masters for Transcendental Meditation, a mental technique which helped me cope with the stress of being a graduate student.

This research was supported by NIH Grant HD 13525 and generous funds from the Department of Zoology, University of Tennessee.

ABSTRACT

The large granulosa lutein cells of the corpus luteum of the ovary in the pig synthesize and secrete progesterone. Receptors that bind luteinizing hormone (LH) and others that bind low density lipoproteins (LDL) located on the outer surface of this cell type play important roles in the regulation of the secretory event. Both types of receptors when bound with their specific ligand have been demonstrated to be internalized by endocytosis. New receptors replace the lost receptors and the process is repeated many times for the life of the cell. Neither the source of new receptors nor the mechanism for the replacement process has been identified.

Earlier studies by the author (Gonadotropin Receptors of the Ovarian Granulosa Lutein Cell of the Pregnant Pig, Master's Thesis, University of Tennessee, June 1982) revealed that a pool of unbound gonadotropin receptors was located within the cell and that subcellular fractions enriched with Golgi body membranes or endoplasmic reticulum possessed gonadotropin receptors, especially the Golgi body fractions. A presupposition was made that the intracellular receptors located in these organelles were the source of new receptors, but the mechanism of transport of these receptors from within the cell to the outer surface was unknown.

Examination of this cell type by electron microscopy revealed that coated vesicles, known membrane transporters within cells, were located near the vicinity of the Golgi body as well as near the surface of the cell. A hypothesis was presented that the coated

vesicles seen at or near the Golgi bodies were responsible for the transport of newly synthesized or recycled receptors in the Golgi to the outer cell surface.

To test the hypothesis, coated vesicles were isolated from these cells using differential and density gradient centrifugation techniques and their receptor content was examined. A fraction of coated vesicles estimated to be greater than 97% pure was obtained. Analysis of the coated vesicle fraction using radioreceptor assays revealed that it possessed receptors for both LH and LDL and thus is consistent with our hypothesis.

PREFACE

The main purpose of graduate study for a Doctor of Philosophy degree in the Department of Zoology at the University of Tennessee, Knoxville, is to train students to be competent research scientists that can make astute observation, formulate a sound hypothesis about the observation, and then conduct decisive experimentation to test the hypothesis. As a means to measure a student's competence, each student is required to perform an original piece of research and to present the research orally and in prose for evaluation. The dissertation presented here is a measure of my success in this area.

The objective of the dissertation research presented here was to study the mechanism of gonadotropin and LDL receptor synthesis and insertion into the plasma membrane of porcine ovarian granulosa lutein cells. Initially, this project started out as my Master's thesis research but because of the complexity of the topic and the constraints of time, I was unable to fully complete the project. Furthermore, as so often happens in Science, for each question answered ten new questions arise. After I completed my Master's, I felt that I knew less about my research project than I did before I started. In an unending battle to quench my thirst for understanding, I have decided to pursue - for my dissertation research - the same topic I chose for my Master's; except this time I am peeling off a more subtle layer of questions as well as providing a few more answers.

The dissertation is divided into four parts. Part 1 is an extended introduction that lays the foundation for the dissertation research presented in the following parts. In this part a general overview of the structure and function of the pig ovary is layed out with a brief review of the Master's research, which as discussed earlier, developed into this dissertation project. Part 2, "The Isolation of Coated Vesicles from the Corpus Luteum of the Pig," discusses the inherent problems with isolating coated vesicles from the corpus luteum and the solution of the problems. Part 3, "Gonadotropin and LDL Receptors are Present within Coated Vesicles Isolated from the Corpus Luteum of the Pig," presents evidence that supports the title. Parts 2 and 3 are written in the format of a scientific paper and therefore each has its own "Introduction," "Materials and Methods," "Results," and "Discussion" sections. Part 4, the last part is a brief summary and conclusion of the dissertation research. A model for the mechanism of gonadotropin and LDL receptor synthesis and insertion is also presented.

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PART 1

INTRODUCTION

As a foundation for the research presented in this dissertation, I think it is imperative that the structure and function of the corpus luteum, my experimental model, be reviewed briefly.

I. GENERAL STRUCTURE OF THE OVARY

The ovaries are approximately the size of shelled almonds with the dimension of 2.5 X 5.0 X 1 cm. They are covered by a simple cuboidal mesothelium. Underneath this layer, the ovary can be divided into an outer cortex and an inner central medulla.

The cortex (zona parenchyma), which is the largest portion of the ovary, primarily consists of a modified connective tissue called the stroma. This tissue consists of closely packed myoid, epithelioid and fibroblast-like cells, collagen fibers and intercellular substance usually arranged together in whorls. Near the surface of the ovary the stroma tissue becomes modified in its arrangement and content to form a thick, dense and continuous layer of collagen fibers and fibroblast-like cells generally referred to as the tunica albuginea. In the mature pig ovary, epithelial follicles of various sizes each containing an oocyte can be found scattered throughout the cortex but always under the tunica albuginea. Depending on the age of the female, there may be as many as several hundred thousand of these follicles per ovary. They range in size from the microscopic, but numerous, primordial follicles approximately 50 um in diameter to the very large preovulatory follicles, 8 - 11 mm in diameter. The different sized follicles represent follicles at various stages of

development. This topic is discussed in more detail later. In addition, the cortex of the pig ovary may contain multiple corpora lutea, a corpus albicans and atretic follicles.

The medulla (zona vasculora) which occupies the center of the ovary consists primarily of a loose connective tissue containing collagen and elastic fibers, true smooth muscle cells, epitheliod and fibroblast like cells. In this part of the ovary, the major blood vessels that enter or leave can be found, as well as lymphatic vessels and nerve fibers.

II. BLOOD AND NERVE SUPPLY

The ovary receives its blood supply from two different arteries, the more important of which is the ovarian artery that derives directly from the abdominal aorta and sends branches to both the ovary and fallopian tube. A uterine artery, which is the major supplier of blood for the uterus and vagina, also has tributaries that intertwine with the ovarian artery. The peculiar intertwining of arteries that feed the ovary, fallopian tube and uterus has been referred to as the genital vascular arcade (176). Blood vessels from both of these arteries pass into the ovary through the hilum into the medulla. Smaller branches of the arteries radiate out centrifugally to the outer cortex where even smaller coiled arterioles form capillary beds in the stroma tissue and in the outer surrounding layer of larger developed follicles. Blood and lymph are drained by vessels that pass out of the ovary along the same route in which arteries entered. A

venous genital arcade is responsible for intertwining of veins draining the gonad, fallopian tube, and uterus. At several places along the veins, the vessel walls are very thin and are closely associated with and coiled about the ovarian artery forming the pampiniform plexus. This unusual arrangement is thought to form a counter current exchange mechanism between the ovarian artery and vein to facillitate the accumulation of a high concentration of ovarian made steroids within the ovary (65).

The ovary is richly innervated with both unmyelinated sympathetic and cholinergic fibers that regulate the blood flow, contractile components of the stroma, and may directly contribute to regulation of steroidogenesis (85, 112).

III. ESTROUS CYCLE

The estrous cycle is characterized by morphological and functional changes throughout the entire reproductive system that repeat in a predictable manner. The mechanism for these cyclic changes are only partially understood but appear to involve an interplay of hormones produced by the pituitary, ovary, and uterus which directly or indirectly affect each other. In the pig the estrous cycle is approximately 21 days and, as for most mammals, is roughly divisible into four stages: Proestrus, Estrus, Metestrus, and Diestrus.

1) Proestrus. During Proestrus, FSH secreted by the pituitary induces follicular development in the ovary. The larger growing

follicles produce estrogen and estrone. The estrogens induce rapid growth in tissues throughout the reproductive system and as levels rise, a positive feedback stimulates the pituitary to produce larger amounts of FSH and LH. When a critical threshold concentration of estrogens is reached in the blood, a temporary surge of both FSH and LH is secreted from the pituitary. This surge of gonadotropins brings about the final maturation of some follicles in the ovary, as well as the onset of the next stage, Estrus. 2) Estrus is characterized by behavioral changes in the pig making her sexually receptive. It is also during this period that ovulation will occur, usually 38 - 42 hours after the onset of estrus. The period following estrus is termed (3) Metestrus. During this period corpora lutea develop from ovulated follicles and begin to secrete copious amounts of progesterone. Progesterone causes the final maturation of the uterus as well as changes in other parts of the reproductive tract. The corpora lutea will persist through Metestrus for about two weeks, after which time, in the pig, the uterus is thought to secrete prostaglandins which are luteolytic and will induce corpora lutea regression. At this time, Metestrus ends and Diestrus begins. Diestrus will last for about 4 days. If pregnancy occurs or if a hysterectomy is performed on the pig, the corpora lutea will persist for about the length of a normal gestation period, 126 days, and then regress (12, 93).

In reference to the ovary, the estrous cycle can also be categorized into the follicular phase (Proestrus and Estrus),

and ovulation and luteal phase (Metestrus and Diestrus). I favor this terminology and will use it throughout the dissertation.

IV. FOLLICULAR PHASE

Although the mechanism that induces follicular development and the biochemical processes involved in developmental changes of follicles is poorly understood, the morphological changes that occur in developing follicles have, on the other hand, been well characterized for many mammals and are described in detail in the literature (12, 29, 45, 56, 89, 105, 107, 126, 134, 147, 148, 163, 218).

Primordial and Primary Follicles

Each ovary, depending on the age of the pig, possesses in excess of several hundred thousand primordial follicles. Each of these follicles consists of a primary oocyte, 25-30 um in diameter, surrounded by a single layer of epithelial pregranulosa cells. The entire structure rests on a thin acellular basal lamina. At the onset of each estrous cycle, FSH released from the pituitary is thought to induce at least 25 - 30 primordial follicles from the resting follicular pool in each ovary to begin a course of development. The most obvious change in the follicle occurs with the granulosa cells. They undergo a rapid proliferation and as their numbers increase, the oocyte becomes completely surrounded by many layers. The entire structure is now encapsulated by a very prominent basal lamina, 1-2 um

in thickness, which is referred to as the glassy membrane. As the follicle continues to grow in size it begins to sink deeper into the cortical stroma. Surrounding stroma cells ensheath the follicle forming the theca follicular layer. The growing follicle is then redirected towards the surface of the ovary by the formation of a wedged shaped thecal cone (197). During this process, the smaller resting primordial follicles are pushed laterally out of the way by the theca cone to allow for rapid expansion of the growing follicle. Meanwhile, the oocyte is also growing in size, the cytoplasmic/nuclear ratio increases and a clear refractile, highly polymerized gel-like membrane, the zona pellucida, forms on the outer surface of the oocyte.

Secondary or Vesicular Follicles

When the follicle reaches a size consisting of about 6 - 12 layers of granulosa cells, small fluid filled spaces begin to form between granulosa cells, the call exner bodies. With time these spaces coalesce as more fluid is added and eventually form a single large fluid filled cavity, the antrum folliculi. During this time the outer thecal layer differentiates into two layers: 1) theca interna which contains steroid secreting cells and a rich capillary and lymphatic network and 2) theca externa which contains a sheath of contractile myoid like cells, collagen fibers and intercellular ground substance.

The follicle has no endocrine function prior to the formation of the antrum, but after this event and with the formation of the theca

interna, the follicle becomes an endocrine gland and secretes estrogens. The concentration of estrogens within the antrum of large follicles can become exceedingly high. In fact, measured levels of estrogen in large preovulatory follicles have been reported for humans to be 40,000 times the level measured in the peripheral blood (139). There is some controversy as to which cell type in the follicle - either thecal or granulosa - is responsible for the synthesis of estrogens (45). Isolated theca cells from some species have been shown to have all the enzymes necessary to make androgens from cholesterol or progesterone, but lack aromatase activity to convert androgens into estrogens. In contrast, isolated granulosa cells do not possess the enzymes, at least not in sufficient quantities, to make androgens, but interestingly, when given exogenous androgens can convert these into estrogens. Further evidence that androgens are taken up rather than synthesized by granulosa cells comes from the observation that when mixtures of thecal and granulosa cells are incubated in the presence of antisera to testosterone the synthesis of estrogen is blocked. Presumably, if androgens and estrogens were synthesized within the confines of the same cell, the antisera would be ineffective (126). On the basis of these observations, a Two Cell Hypothesis is favored by many to explain the high rate of estrogen synthesis (24, 62, 71, 72, 117, 123, 128). It states that the thecal cells synthesize androgens from cholesterol which are transported into the follicle where the granulosa cells take up the androgen and convert it into estrogens. Since granulosa cells are devoid of

capillaries, the estrogens then have to be transported back to the theca for secretion into the blood stream.

Preovulatory or Graafian Follicles

Towards the end of the follicular phase after the surge of gonadotropins is released from the pituitary, approximately 5 - 10 large preovulatory or graafian follicles can be found in each ovary of the pig. They range in size from 8 to 11 mm and very precariously bulge out from the surface of the ovary. Within these giants, the oocyte has matured to its full size of 125-130 um in diameter. It remains attached to the inner wall of the follicle by a stalk of epithelium, the cumulus oophorus that is continuous with a sheath of granulosa cells that intimately surround the oocyte, the corona radiata. The oocyte will resume meiosis, complete the first meiotic division and is again arrested at Metaphase II until after ovulation.

The antrum of the follicle continues to accumulate fluid which contains transudate from plasma and secretions from follicular cells. The fluid contains inorganic ions present in concentrations similar to those found in plasma, a variety of proteases, glycosaminoglycans such as hyaluronic acid, several proteins as follitropin and steroid binding protein and high concentrations of estrogens, androgens and progestrins. As the follicle enlarges, it pushes against the follicle wall and causes the granulosa and theca layers to thin out.

A cross section through the wall of a graafian follicle is shown in Figure 1. The different strata are clearly seen. The first layer from the antrum out is the granulosa follicular membrane. Through

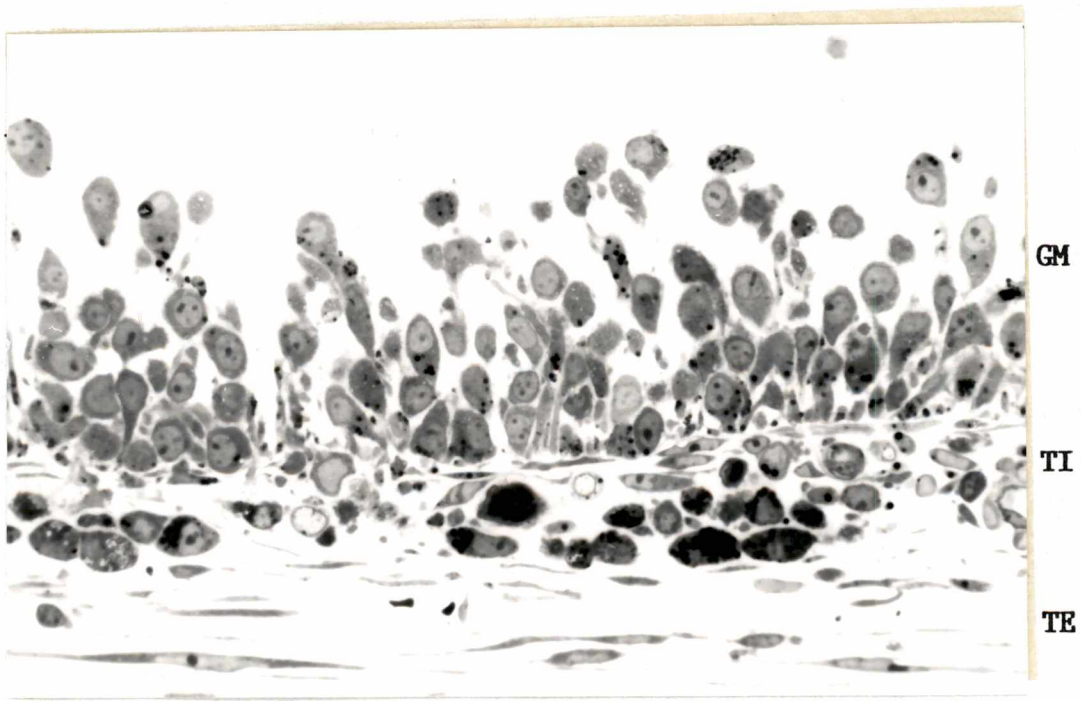

FIGURE 1. Light micrograph of cross section through the wall of a graafian follicle. GM, granulosa membrane; TI, theca iterna; TE, theca externa. Magnification 528x.

serial sectioning we have determined that in the pig this layer is composed of a pseudostratified epithelium. Every cell appears in some way to connect to the outer glassy membrane. The next layer is the theca follicular layer. The theca can be structurally and functionally subdivided into the theca interna (TI) consisting of ovoid steroid secreting cells and the theca externa (TE) consisting of contractile myoid and fibroblast like cells. Figure 2 shows a thin section through a granulosa follicular cell. This cell, 10-15 um in diameter, has a large nucleus/cytoplasm ratio, a well-developed Golgi body, scanty amounts of endoplasmic reticulum and only a few mitochondria that appear to have shelf cristae. In Figure 3, theca interna (TI) cells can be seen which obviously can be distinguished from granulosa cells by their large lipid inclusions (LI).

V. OVULATION

A variety of factors appear to contribute concomitantly to the ovulatory process (125, 134). This event is characterized by a substantial increase in blood flow to the ovaries (68). The vessels that supply the preovulatory follicle dilate and the capillary becomes more permeable. The glassy membrane surrounding the follicle begins to fragment and for the first time large plasma proteins and blood cells including thrombocytes enter the follicular antrum (68). Hydrolases already present in the follicle as well as those that are newly secreted from either granulosa cells or invading blood cells become activated. As a consequence of the breakdown of the large

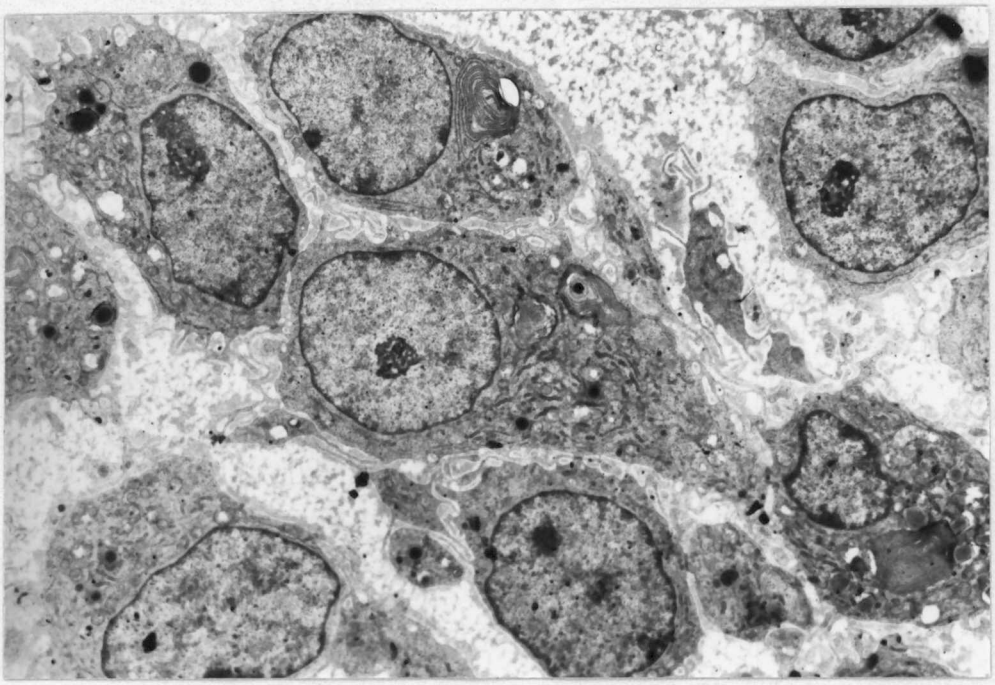

FIGURE 2. Electron micrograph of a follicular cell. Magnification 4,400x.

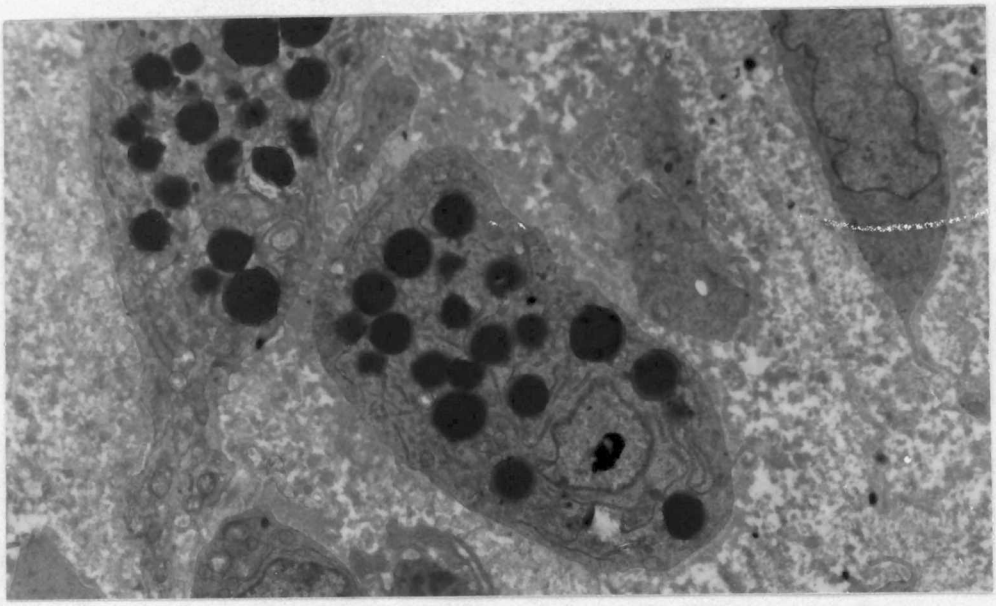

FIGURE 3. Electron micrograph of a theca interna cell. Magnification 5,520x.

glycosaminoglycans, the viscous follicular fluid is converted into a thinner watery fluid. The increased number of smaller soluble molecules within the cavity also has an osmotic effect which draws additional transduate from the surrounding capillaries. The granulosa cells at this time also greatly increase their secretions and contribute to the antral fluid resulting in a rapid enlargement of the follicle and flattening and thinning out of the granulosa membrane. Contrary to early beliefs, careful measurements have indicated that the intrafollicular pressure does not increase during ovulation, probably because of compensatory changes in the follicular wall (125). During this time many of the granulosa cells separate from each other and the ovum also detatches from the inner follicular wall. A weak spot forms in the follicular wall at the site where the ovum will be extruded. Although the mechanism for this is not fully understood, studies carried out by McClellan and Abel have demonstrated using cytochemistry for acid phosphatase that the granulosa cells bordering the tunica albuginea posses lysosomes that appear to be released to the outside of the follicle where they can digest the tunica albuginea. The weak spot or thinning out of the tunica albuginea can be visually observed on the outer surface of the ovary as a growing and protruding red spot, the stigma. During the actual ovulatory event, the follicle ruptures through the ovarian wall at the exact spot where the stigma can be seen. Concomitant with this, the theca externa contracts and the ovum, along with follicular fluid, is slowly extruded out of the follicle to be captured by the fallopian tube.

VI. LUTEAL PHASE

Formation of the Corpus Luteum

Immediately following ovulation with the release of follicular fluid, the follicle collapses, the follicle wall folds upon itself and the follicular cavity fills with blood and clots. With the disruption of the glassy membrane, blood vessels sprout and invade along with connective tissue and theca cells. Over the next several days the follicle will undergo a remarkable transformation to form the corpus luteum (C.L.) (1, 2, 12, 28, 29, 43, 45, 56, 66, 74, 75, 87, 89, 93, 105, 107, 126, 134, 147, 148, 163, 218).

The chief event in the transformation is the cytomorphosis of the granulosa cells. They are exposed now for the first time to a capillary circulation. In the rich nutrient milieu, the cells hypertropy from 10-15 um to 30-40 um in diameter. The cytoplasm/nuclear ratio increases, extensive pleomorphic folds on the plasmalemma form and increase the total surface area. Many well-developed Golgi bodies form. Mitochondria increase in number, elongate and develop tubular cristae. Abundant cisternae of endoplasmic reticulum can now be found throughout the cytoplasm and large lipid inclusions develop in these cells. Many of these lipid inclusions have sworls of endoplasmic riticulum wrapped around them. The end result of the cytomorphosis is to produce a steroid secreting cell. This cell now called the granulosa lutein begins to secrete copious amounts of progesterone and 17a hydroxy-progesterone.

If pregnancy occurs, the corpus luteum (C.L.) will undergo further morphogenesis and become the corpus luteum of pregnancy. In the pig the granulosa cells will enlarge slightly to about 40 um. The ultrastructure of these cells is similar to that before pregnancy except that increasing numbers of secretory granules can be found in the cytoplasm as the gestation proceeds up to about 16 hours prior to parturition at which time few secretory granules remain in the cells, Figure 4. Using immunocytochemistry, Kendall (113) has demonstrated that these granules contain relaxin hormone. Moreover the depletion of secretory granules in these cells prior to parturition coincides with rise in relaxin levels measured in the main circulation (12, 13, 27, 28).

A thick section through the corpus luteum of pregnancy of mid-gestastion is shown in Figure 5. A connective tissue capsule (cp) consisting of dense fibrous connective tissue forms the outer limits of the corpus luteum. Finer connective tissue strands follow the plication in towards the center of the corpus luteum (C. L.) where delicate reticular fibers radiate out into the parenchyma to form a supportive meshwork. Blood vessels follow the C. T. framework into the C. L. and form an elaborate capillary network surrounding each cell. The parenchyma appears to be composed of two types of cells, large and small granulosa lutein (56). The large granulosa are more prevalent than the small granulosa during the gestation period. Even though these granulosa cells differ in their ultrastructure (156), both types have receptors for LH and secrete progesterone (121, 122)

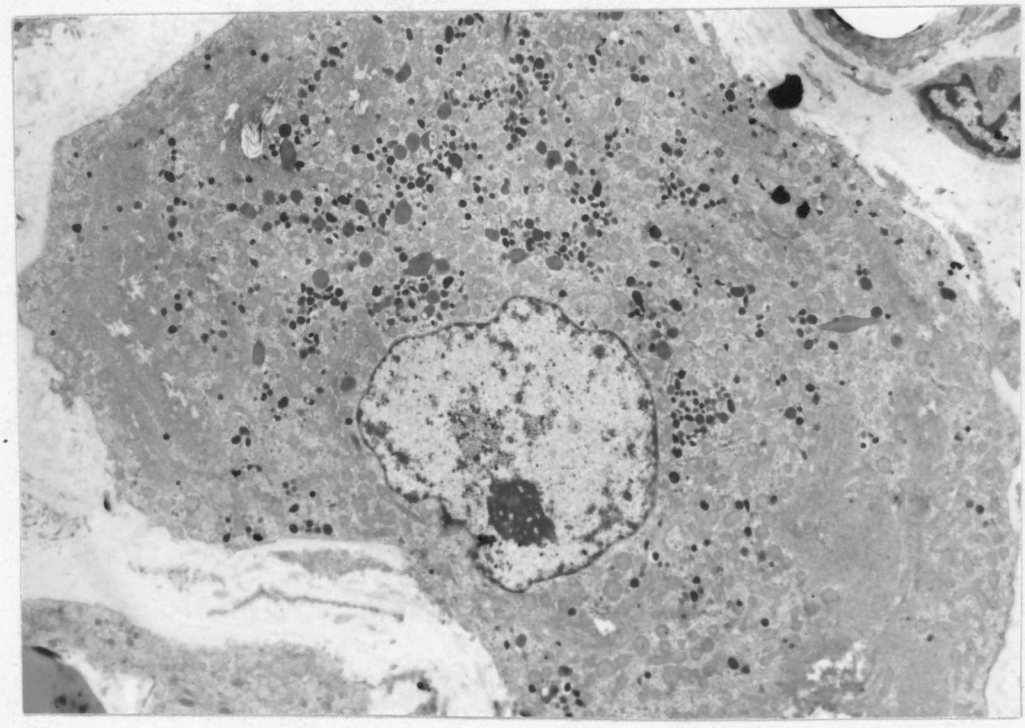

FIGURE 4. Electron micrograph of a granulosa lutein cell. Magnification 4,400x.

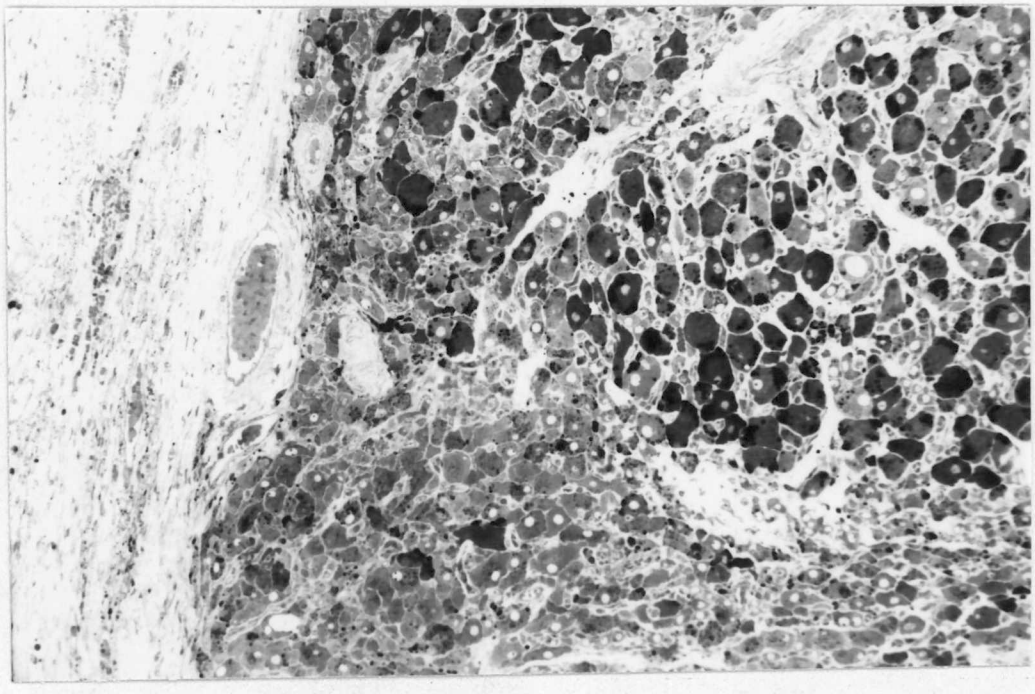

FIGURE 5. Light micrograph of the porcine corpus luteum of pregnancy. Magnification 132x.

and 17a hydroxyprogesterone (185). Although still controversial, it is thought that in the pig the large granulosa lutein cells are derived from granulosa follicular cells whereas the small granula lutein are really theca lutein cells derived from the theca interna cells (56). Theca lutein cells are most easily identified in areas of the C. L. directly under the C. T. capsule.

Morphometric analysis of the numbers, volumes, and types of cells present in the corpus luteum of the sheep were determined by O'Shea (156). The large granulosa lutein cells constitute only about 5% of the total number of resident cells, but they can, on the other hand, constitute approximately 80% of the total volume of the corpus luteum. In this regard, the large granulosa lutein cells are the principal cell type of the corpus luteum.

Luteal Regression

If pregnancy does not occur, although it usually does in the pig, the corpus luteum will begin to regress within two weeks following ovulation to form a new structure, the corpus albicans (1, 2, 12, 29, 43, 45, 56, 66, 89, 93, 105, 107, 126, 134, 136, 147, 148, 158, 163, 183, 218).

In the pig and other large domestic animals, prostaglandin (PGF_2a) secreted by the uterus is thought to be the major luteolytic inducer (23, 78, 106, 151, 166, 216, 217). For example, PGF_2a injected in vivo into ewes rapidly brings about luteal regression (136, 88). In the pig, if the uterus (the major source of luteolytic prostaglandins) is removed prior to the onset of luteal regression,

the corpora lutea are maintained for a period exceeding that of gestation (12, 13, 50).

At the onset of luteal regression the blood flow to the ovary and corpora lutea drops markedly. Prostaglandins which can be measured in high concentration in the plasma of the pig at this time, are potent vasoconstrictors (216, 217, 166) and may in part be responsible for luteolysis. Prostaglandins decrease the permeability of capillaries (162, 151) and also appear to have a direct effect on granulosa cells in which physical properties of membranes including human chorionic gonadotropin (hCG) binding are altered (40).

Changes within granulosa lutein cells associated with regression include disruption of smooth endoplasmic reticulum, decrease in the density and number of mitochondria and a decrease in the progesterone synthesis. Other changes include a dramatic increase in the number of lysosomes and the appearance and accumulation of double-walled autophogocytic vacuoles. The nucleolus becomes less prominent and eventually the nuclear material condenses and the nucleus becomes pycnotic. The cell dies and fragments.

As the regression of the C. L. continues more granulosa cells die, macrophages invade and phagocytose cell debris, and there is a gradual increase in connective tissue elements which will eventually completely replace the granulosa cells.

VII. RECEPTORS OF GRANULOSA LUTEIN CELLS

The large granulosa lutein cells of the corpus luteum of the pig synthesize and secrete copious amount of progesterone and 17a hydroxy-progesterone. Receptors that bind leutinizing hormone (LH) and others that bind low density lipoproteins (LDL) found in this cell type play important roles in the regulation of the secretory event.

Gonadotropin Receptors

The granulosa lutein cell in all species studied has been shown to contain large numbers of receptors localized on the plasma membrane that bind LH or hCG (3, 7-10, 17, 19, 20, 30, 31-33, 46, 47, 49, 59, 64, 86, 91, 94, 109, 110, 116, 119, 124, 164, 165, 169, 170, 180). Although the total number of these surface receptors has not been accurately determined, there may be as many as 60,000 or as few as 3,000 per cell as estimated for corpora lutea in other species (61, 86).

Binding of gonadotropin, the major secretagogue for this cell type, to its receptor initiates an activation of the cell (42, 52, 55, 58, 60, 63, 94, 97, 100, 119, 182); involving production of cyclic nucleotides (6, 42, 101, 118, 131-133, 179, 200, 201), calcium transport (175, 206) and probably other local ionic and pH changes in the membrane (99). This activation process leads to an increase in the metabolic activity within the cell and ultimately to an increase in the production and secretion of progesterone both in vivo and in vitro (3, 21, 22, 61, 98, 108, 133, 185, 186, 188, 195).

The gonadotropin receptor complex has a half-life of between 8-24 hours even though the hormone only effectively activates the metabolism of the cell at the initial time of binding (3, 49, 143). A small amount of the hormone probably dissociates from the receptor after binding (19, 67, 181), but most of the hormone appears to be degraded within the luteal cell itself. The hormone-receptor complexes aggregate into patches along the cell surface (3, 10, 48) where they are taken up by endocytosis (3, 5, 17, 49, 53, 55, 58, 82, 91, 92, 124, 141, 149, 164, 165, 174, 179). In the bitch, ewe, rat, and pig it is clear that patching and endocytosis involves intimate association with the coated pit and coated vesicle system respectively (1-3, 7-10, 17, 82). Following internalization, the endocytotic vesicle fuses with secondary lysosomes, and the hormone (3, 48, 49, 53) and possibly the receptor (18) are rapidly degraded. A single class of secondary lysosomes, the multivesicular bodies, appears to be the organellar system primarily responsible for metabolizing the hormone (3, 17, 18, 48, 49, 77, 91, 202).

LDL Receptors

The surfaces of granulosa lutein cells also possess receptors that bind LDL (11, 21, 22, 36, 50, 102, 115, 153, 161, 170, 190, 207, 208). Although the exact number of these receptors has not been determined for this cell type, in fibroblast, the numbers may vary considerably depending on the physiological state of the cell.

Binding of LDL, the major source of cholesterol for steroid synthesis (11, 21, 22, 90, 198), to its receptor initiates an

internalization of the LDL-receptor complex (14, 15, 35, 38, 39, 78, 80, 82, 82, 154, 161). Within the endosome compartment of the cell, the LDL dissociates from its receptor and is channeled to the lysosome for degradation. The receptor appears initially to escape degradation and is recycled to the cell surface for reutilization many times over (25, 37, 38, 39, 82). However, eventually even the receptor follows the same fate as LDL and is destroyed in the lysosomes (25, 37, 38, 39, 82). The half life for the LDL receptor in fibroblast is estimated to be 30 hours (37, 38, 80).

The cholesterol released from LDL degraded in lysosomes is the major source for the synthesis of steroids (11, 21, 22, 90, 198, 205). Cholesterol also serves as a second messenger and has three major effects on cholesterol metabolism: 1) HMG-CoA reductase, a key regulatory enzyme in the cholesterol synthetic pathway, is suppressed (76, 127). 2) Acyl CoA: cholesterol acyltransferase (ACAT), a cholesterol esterfying enzyme, is activated so that excess cholesterol can be stored in the cytoplasm as cholesterol ester (lipid) droplets (79). 3) The synthesis of new LDL receptors is suppressed so that the cell can adjust the number of LDL receptors to provide only a sufficient amount of cholesterol without an excess accumulation (34, 184). Through these mechanisms the cell is able to maintain a constant level of cholesterol for steroid synthesis despite the fluctuations in exogenous sources.

Regulation of Receptors

Granulosa lutein cells become "down regulated" when exposed to large doses of gonadotropins. "Down regulation" is characterized by an almost total loss of gonadotropin receptors from the cell surface and a refractory period where adenylate cyclase is desensitized and almost no progesterone is secreted from the cell (10, 17, 54, 95, 101, 103, 174, 177, 192, 193, 215). Gradually with time however the cells regain their capacity to bind gonadotropins and secrete progesterone (61, 95, 104, 141, 199). These data plus the fact that luteal cells can remain functionally active from a period of several weeks (normal estrous cycle) to several months (pregnant animals) (12, 43, 66, 93, 105, 126, 147, 148, 163, 218) indicate that there must be some internal cytoplasmic mechanism for replacing receptors lost from the cell surface (3, 114).

The studies performed for my Master's Thesis research indicated that there is an abundant source of "hidden" unbound gonadotropin receptors within the cytoplasm, particularly the Golgi body and rough endoplasmic reticulum of the granulosa cells. This finding is in perfect agreement with those performed on the corpus luteum in the cow (142, 172) or rat (31, 32). Considering the substantial documentation on membrane flow from the RER-Golgi-PM axis and the gradual differentiation of the constitutive enzyme components in each compartment (69, 73, 111, 140, 144, 145, 146, 203) it seems likely that the large pool of "hidden" receptors in the Golgi region could serve as the source of new receptors on the cell surface. It is

unclear, however, whether the "hidden" receptors in the Golgi are derived from receptor recycling through the lysosomal agranular ER system or are the result of new synthesis in the RER and Golgi or both.

How then are the "hidden" receptors in the Golgi body transported to the cell surface? After ovulation the small granulosa cells which surround the graffian follicle progress through a period of extremely rapid growth and differentiation (2, 66, 138). Gonadotropin receptors (LH-hCG) become abundant on the cell surface within the first 24 hour post-ovulatory period (46, 47, 135, 149, 169, 177). Receptor appearance can be correlated with the formation of surface microvilli (32, 44, 130), and the development of small coated vesicles at the perimeter of the Golgi elements and formation of coated caveolae along that portion of the plasma membrane lined with the newly formed microvilli (1, 2, 3, 138). In vivo, tracer studies performed in this laboratory using tritiated fucose and autoradiography in the dog and ewe, indicated that the small coated vesicles move in mass towards the cell surface and become incorporated into the plasma membrane during this period of development (1, 2, 3, 138). We hypothesized therefore that they might be the primary vehicle by which the newly synthesized gonadotropin receptors were inserted into the plasma membrane.

Similar autoradiographic tracer studies carried out after the granulosa luteal transition was complete,however, revealed no such migration of the small coated vesicles (2, 3, 138). In fact, the number of coated vesicles in the perimeter of the Golgi region was

reduced. During the luteal phase of the cycle abundant secretory granules were formed at the level of the Golgi in cells activated by gonadotropins. These granules moved rapidly towards the cell surface where the membrane of the secretory granule fused with the plasma membrane during exocytosis (3, 27, 51, 57, 74, 75, 159, 160, 187, 188). Very few granules were ever observed in unactivated cells, so there appeared to be little or no storage of the granules or their contents. In other words, activated granulosa lutein cells during the midluteal phase of the cycle were active in both endocytosis and exocytosis.

On the basis of these observations, a new hypothesis was formed that the membrane surrounding the secretory granule was the vehicle by which new receptors were inserted into the cell surface during the luteal phase of the cycle and pregnancy. My Master's Thesis research ("Gonadotropin Receptors of the Ovarian Granulosa Lutein Cell of the Pregnant Pig," University of Tennessee, June 1982) indicated that this primary hypothesis was incorrect. Secretory granules isolated from the corpus luteum to near homogeneity were examined for their content of hormone receptors. No gonadotropin receptors were found and therefore this organelle is probably not involved in the transport of newly synthesized or recycled receptors in the Golgi body to the outer cell surface for insertion.

The mechanism in which gonadotropin receptors, or any receptor for that matter, are transported from the Golgi body to the plasmalemma in the granulosa lutein cell is still unknown. Our new

hypothesis is that the coated vesicles located in the vicinity of the Golgi body, although few in number, are the vehicle for receptor transport. The purpose of the research presented in this dissertation was to test this primary hypothesis.

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PART 2

ISOLATION OF COATED VESICLES FROM THE CORPUS LUTEUM OF THE PIG

I. INTRODUCTION

Coated vesicles first described by Roth and Porter (1964) have been found in all Eukaryotic cells studied to date (63). They have been implicated in many cellular processes that involve membrane surfaces: receptor mediated endocytosis (1, 2, 12, 17, 18, 22, 23-25, 27, 40, 44, 47, 51-53, 57, 61, 63, 64, 68, 72, 75, 76, 78-81), membrane recycling (16,24, 29, 30, 57, 64, 72), intracellular protein transport (21, 26, 40, 52, 57, 64) and in exocytosis of both membrane and secretory proteins (13, 18, 20, 21, 35, 41, 63-66).

Coated vesicles as described by Kanaseki and Kadota (1969) consist of a membrane vesicle surrounded by an icosohedral lattice work of protein (34). Pierce (14, 54, 55) showed that the characteristic coat structure is made up primarily of one protein called clathrin. The basic assembly unit for the coat structure appears to be a larger multimeric protein made up of three molecules each of clathrin and 100 K dalton protein that are joined together to a central locus to form a three armed structure, the triskelion (42, 74, 75). Triskelions are then assembled to form the protein lattice work of pentagons and hexagons characteristic of the coat structure (14, 15, 18, 31, 32, 34, 36, 56). Recently other proteins have been found associated with coated vesicles that direct the rate of assembly (33, 38, 39, 59, 62, 73), final size (59, 62, 82) and disassembly (7, 69, 70) of clathrin coated vesicles.

The elucidation of the structure and partial understanding of the functional roles coated vesicles play in cells was made possible by

the studies performed on isolated coated vesicles that were derived from porcine brain (54, 55). The basic procedures used for the isolation of coated vesicles have been based primarily on the original procedure developed by Pierce (54, 55) for Pig brain. This procedure requires large quantities of tissue, takes several days to complete, and produces a small yield. Although quite sufficient for porcine brain and some other tissues, the procedure is not, however, suitable for the corpus luteum of the ovary for several reasons: 1) Granulosa lutein cells possess large numbers of multivesicular bodies (lysosomes), rich in hydrolytic enzymes that rupture easily by either mechanical agitation or osmotic shock and, consequently, released proteolytic enzyme contaminate the fractions. 2) The cytoplasm of this cell type is completely filled with many sworls, sheets and tubules of endoplasmic reticulum. During typical homogenization procedures these membranous structures fragment and reseal to form many small membrane vesicles about the same size as the coated vesicle. These small membrane vesicles greatly outnumber the coated vesicles and interfere severely with the isolation of coated vesicles.

The procedure outlined in this paper is more suitable for isolating coated vesicles from the corpus luteum. It has several advantages over the basic Pierce's method. It requires less tissue, is designed to eliminate osmotic or proteolytic damage to coated vesicles and other organelles, circumvents the problem of small membrane vesicles and their aggregates, and can be completed within one day.

II. MATERIALS AND METHODS

Material

All chemicals used in this study were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium Iodide-125, carrier free, was purchased from ICN Biomedicals Inc., Irvine, CA. All tissue used for experimentation were obtained from Lay's Meat Packing Co., Knoxville, TN.

Isolation of Coated Vesicles

The purification scheme for isolation of porcine corpus luteum coated vesicles is summarized in Figure 1. All steps in the isolation procedure were performed at 4°C except where noted. Pig ovaries were collected from the slaughterhouse in ice cold saline solution, and quickly brought back to the laboratory for further processing. Corpora lutea were excised from the ovaries and any extraneous connective tissue was removed. The tissue was minced using a razor blade and placed in buffer A (10 mM HEPES, pH 7.0, 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$, 0.02% sodium azide, 0.1 mM phenylmethyl sulfonyl fluoride [PMSF], 40 ug/ml soybean trypsin inhibitor, and 20 ug/ml tosyl-L-lysine chloromethyl ketone [TLCK]). The volume was adjusted with buffer A to 30% w/v. Typically, 10-100 gm of tissue were used. Homogenization was accomplished using a 40 ml Dounce tissue homogenizer. Tissue was first ground using 6 strokes of the loose fitting pestle. The homogenate was then filtered through 3 layers of gauze to remove connective tissue sheets, followed by further

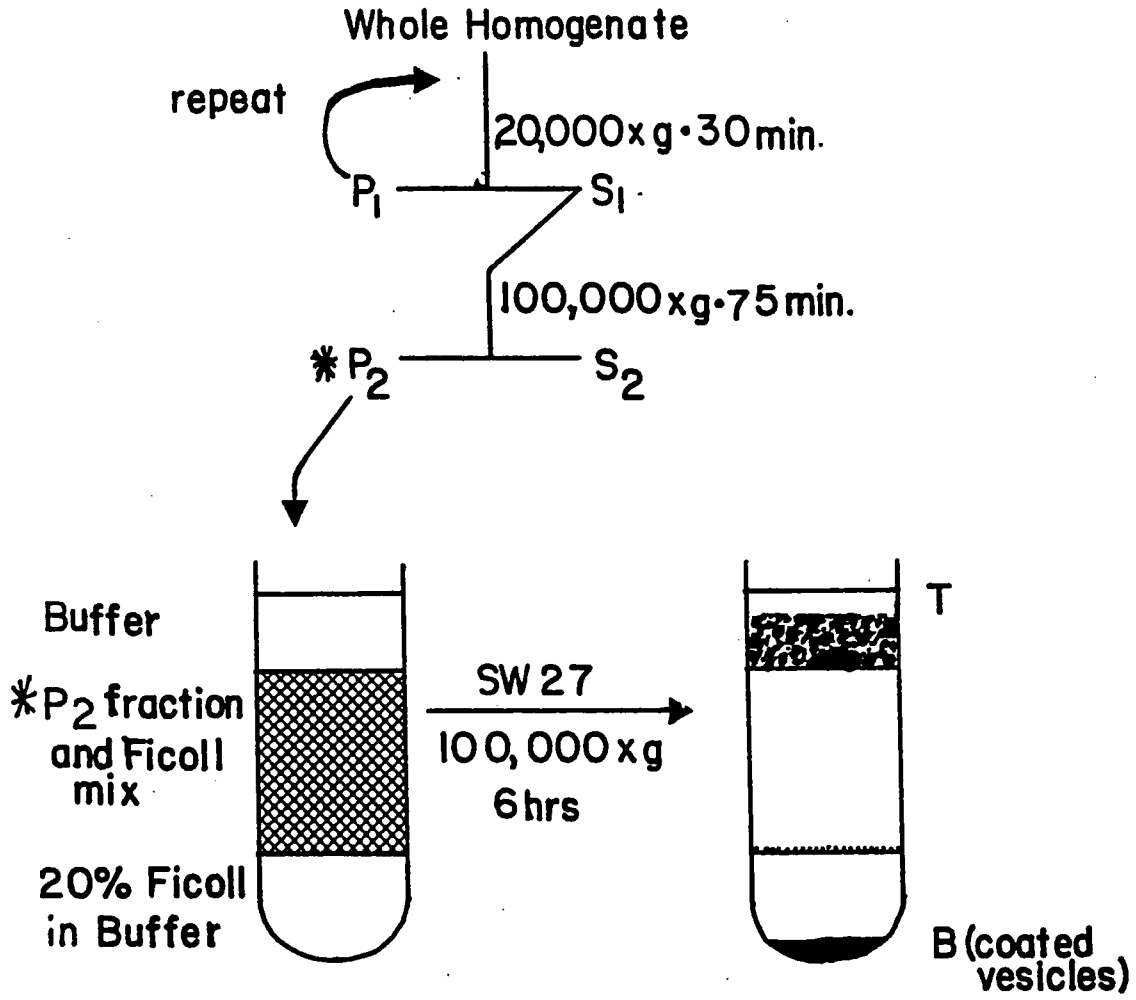

FIGURE 1. Flow diagram of differential and density gradient centrifugation.

homogenization using 15 strokes of the tight fitting pestle. The homogenate was centrifuged at 20,000xg for 30 min in a Beckman J-21C preparative centrifuge fitted with a JA-20 rotor. The supernatant (S_1) was poured off and saved. The pellets (P_1) were rehomogenized in buffer A (half or original volume) and recentrifuged. The resulting supernatant S_1* was combined with S_1. The total supernatant was then centrifuged at 100,000xg for 75 min in a SW27 rotor with the Beckman L5-50 ultracentrifuge. The 100,000xg pellets P_2 were pooled and rehomogenized in buffer A with the Dounce homogenizer using 20 strokes of the tight fitting pestle. The P_2 suspension (26mg protein per ml) was mixed with half as much of a 36% w/v Ficoll$_{70}$ solution containing buffer B (10 mM HEPES, pH 7.0, 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$, 0.02% NaN_3), and rehomogenized as before. The final Ficoll concentration was 12% w/v.

Typically, 15 ml of the P_2-Ficoll suspension was layered on top of a 10 ml solution of approximately 20% w/v Ficoll$_{70}$ in buffer B (refractive index 1.36275) in an SW27 centrifuge tube and this was overlayed with buffer B to fill the tube. The step gradient, Figure 1, was centrifuged at 100,000xg for 6 hrs at 15°C in a SW27 rotor. Two fractions were collected: a pink top layer (T) and an orange pellet (B). Both fractions were diluted at least 5 fold with buffer B and centrifuged at 100,000xg for 75 min at 4°C. The fractions were resuspended in buffer B and rehomogenized in a 7 ml Dounce tissue homogenizer using 30 strokes of the tight fitting pestle. Coated vesicles were stored at 4°C for up to two weeks without any apparent

change in structure or biochemical activities, although aggregates will occur with time and rehomogenization is needed for a homogeneous suspension.

Enzyme Assays

Activities for the marker enzymes, thiamine pyrophosphatase (EC 3.6.1.1) and NADH cytochrome C reductase (EC 1.6.99.3) were measured using appropriate controls by the methods of Bramley and Ryan (8) with slight modifications. Na^+-K^+ ATPase (EC 3.6.1.3) according to a modification of the method by Wallach and Kamat (77). Optimum conditions of pH and substrate concentrations were determined for each enzyme using the whole homogenate. Enzyme assays were performed as three point fixed time assays except for NADH cyto C Red which was a continuous time assay. In either case only the linear reaction rates were used to calculate enzyme activities. The activities were expressed as the quantity of product (nanomoles) formed per min per mg of protein. Protein was estimated by the method of Lowry (46) using bovine serum albumin as a standard. Modifications made in the marker enzyme assays are listed below.

Na^+-K^+ ATPase

The total ATPase assay was run in a 2 ml medium containing 10 mM TRIS maleate, pH 8.0, 0.5 mM Na_2 EDTA, 1 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 2 mM ATP, 0.01% saponin and 100 ul of sample. Control tubes contained similar medium except there was no K^+ and in addition contained 1 mM Ouabain. After incubation at 37°C, aliquots (0.5 ml)

of reaction mixture were removed at 15, 30 and 45 min and stopped by injecting into another tube containing 0.5 ml of ice cold 10% trichloroacetic acid. The precipitated protein was removed by centrifugation at 2,000xg for 15 min in a Beckman table top centrifuge TJ-6R. Aliquots of the supernatant were removed and measured for the presence of free phosphorous according to the method of Fiske and Subbarow (19). The activity of (Na^+-K^+)ATPase was calculated as the difference between reaction rates in the absence and presence of Ouabain.

Thiamine Pyrophosphatase

A 2.0 ml system containing 5 mM Tris-Maleate, pH 7.6, 5.0 mM $MnCl_2$, 3.3 mM thiamine pyrophosphate (cocarboxylase), 0.01% saponin, and 100 ul of sample was used. After incubation for the allotted time point, aliquots were removed, reactions were stopped by ice cold TCA and processed as described above for the Na^+-K^+ATPase assay. Aliquots of supernatant were assayed for free phosphorous using the Bernenblum and Chain method as modified by Martin and Doty (45).

NADH Cytochrome C Reductase

The only modification in the assay was that the final concentration of $MgCl_2$-$2H_2O$ was 1 mM, instead of 2 mM as specified by Bramley and Ryan (8).

SDS PAGE

SDS discontinuous buffer gel electrophoresis was performed using a modification of the system described by Laemmli (43) with 3%

stacking and 10% separating acrylamide gels. The electrode buffer and gels all contained 2 mM Na_2EDTA. Samples (100-200 ug of protein) were prepared by boiling for 5 minutes in sample buffer containing in final concentration 0.0625 M TRIS-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 2 mM Na_2EDTA and bromophenol blue. The samples were subjected to electrophoresis under constant current at 10 mAmp per tube for 6-8 hrs. After which, the gels were fixed and stained for 2 hrs in 0.2% Coomassie blue (R250)-50% Methanol-10% ascetic acid and then destained in 25% methanol-10% ascetic acid overnight.

Electron Microscopy

Thin sections: Small pieces of pelleted fractions were removed with a spatula and were fixed overnight at 4°C in 2.5% gluteraldehyde containing 5% sucrose and 0.1 M Na Cacodylate, pH 7.2. The samples were washed in 0.1 M cacodylate, pH 7.2, containing 5% sucrose, post-fixed for 1 hr in 1% OsO_4 in 0.1 M cacodylate, pH 7.2, stained in block for 2 hrs in 2% uranyl acetate in Maleate buffer, dehydrated in ethanol and embedded in epon. Thin sections of samples cut with a diamond knife on a Riechert ultramicrotome, were stained in uranyl acetate and lead citrate and viewed in a Hitachi H600 electron microscope.

Negative staining: For negative staining, samples diluted with buffer B (approx. 0.5 mg of protein/ml) were deposited on formvar and carbon-coated copper grids for 1 min, the excess sample was removed and the grids were washed with several drops of 2% uranyl acetate in

distilled H_2O. The grids were then stained for 30 seconds with the uranyl acetate solution after which the excess was removed by drawing off with filter paper and then allowed to air dry. Grids were viewed in the Hitachi H600 or H800 at 100 K volts.

Radiolabelling Coated Vesicles

Coated vesicles were radiolabelled with ^{125}I by a modification of the Bolton and Hunter method (5). To a 1.5 ml polypropylene microfuge tube 50 ul of 3-(4-hydroxyphenyl)-propionic acid N-hydroxysuccinimide ester in benzene (2.5 ug/50 ul) was added and allowed to dry under vacuum. Next, on ice, to this tube, 400 ul of a coated vesicle suspension in 20 mM HEPES buffer, pH 7.0 (approx. 200 ug of protein) was added along with 0.2 mCi NaI-125 and the solution was mixed. The labeling was initiated by adding 25 ul of chloramine T (0.5% in 20 mM HEPES, pH 7.0). The reaction was stopped after 10 sec by adding 50 ul of metabisulfite (6% in HEPES) and 500 ul of NaI (7.5% in HEPES). The free iodide-125 was removed from the bound ^{125}I by dialysing the sample against 2 liters of buffer B.

Procedure for Determining the Yield of Recovery of Coated Vesicles

Coated vesicles labeled with I-125 in the above procedure (6.8 x 10^6 cpm) were added to 60 ml of a corpora luteal homogenate. The homogenate was then processed according to the isolation procedure for coated vesicles as described above. Small aliquots were removed from supernatants and pellets in each step of the procedure and their radioactivity was determined by a Beckman gamma 4,000 counter.

III. RESULTS

Isolation Procedure

A critical factor for the isolation of coated vesicles was the use of the proper buffer. In earlier attempts, the standard .1M MES buffer, pH 6.5 containing 1 mM EGTA and 0.5 mM $MgCl_2$ was used. This buffer worked fine in the procedure for the isolation of coated vesicles from brain tissue (14, 54, 55) but not on corpora luteal tissue. No structures recognizable as coated vesicles (CV) could be found in the homogenate, pellets or supernatants. In fact, even when vesicles isolated from the brain were mixed with the supernatant from the corpus luteum homogenate there was significant degradation of the brain CVs within an hour. An assumption was made that degradation could be due to acid hydrolysis since the supernatant contained high levels of activity for both acid phosphatase and B-glucoronidase (data not shown) major marker enzymes for lysosomes. A 10 mM HEPES buffer where the pH was raised to 7.0-7.1 was therefore used in an attempt to reduce lysosomal enzyme activities (optimum activity at pH 5.0). A pH of 7.4 or higher could not be used because at this pH clathrin will dissociate from the coated vesicles (50, 83). To reduce possible osmotic effects on coated vesicles and lysosomes, the osmolarity of the buffer was adjusted to approximately 300 mOsm using NaCl, final concentration was 0.15 M. Even with these two modifications there was still significant degradation of coated vesicles and so the protease inhibitors; phenylmethyl-sulfonyl fluoride (PMS), soybean trypsin

inhibitor and tosyl-L-lysine chloromethyl ketone (TLCK) were added to the buffer. The final buffer used in the homogenization procedure was 10 mM HEPES, pH 7.0, containing 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$, 0.02% NaN_3, 0.1 mM PMSF, 40 ug/ml trypsin inhibitor and 20 ug/ml TLCK. With this HEPES buffer and its additives, the morphological and biochemical integrity of coated vesicles derived from the corpus luteum were effectively maintained.

The isolation procedure was divided into two parts: differential centrifugation and density gradient centrifugation. Figure 1, page 51, shows a summary of the two steps used in differential centrifugation. Table 1 shows activities of marker enzymes for the three most likely membrane contaminants of the coated vesicle fractions; endoplasmic reticulum, NADH Cytochrome C Reductase; Golgi membrane, thiamine pyrophosphatase; and plasma membrane, Na^+-K^+ATPase. The P_1 fraction contained 42% of the total protein, 30% of the total thiamine pyrophosphatase activity, approximately 50% of the NADH Cyto. C Red. activity, and 50% of the total phosphatase activity. Electron micrographs taken of thin sections through this fraction revealed that it contained connective tissue sheets, unbroken cells, nuclei, mitochondria, lysosomes, secretory granules and large membrane vesicles. The P_2 fraction possessed 20% of the total protein, 66% of TPPase activity, 50% of NADH Cyto. C. Red. activity and a small amount of phosphatase activity. Electron micrographs of the P_2 fraction, Figure 2(a), demonstrate that this fraction contained primarily membrane vesicles of various sizes and coated vesicles, although very

TABLE 1. Marker enzyme activities for subcellular fractions. SA, specific activity (enzyme activity [nmole of product/min] divided by mg of protein); EF, enrichment factor (specific activity of fraction divided by specific activity of whole homogenate). (*), unable to determine enzyme activity due to high non-specific phosphatase activity.

	Protein			(Na^+-K^-) ATPase			Thiamine Pyrophosphatase			NADH cytochrome C Reductase		
Fraction	mg/ml	Total	% Recovery	SA	Total	EF	SA	Total x 10^3	EF	SA	Total x 10^3	EF
WH	21.1	6,346	100	*	-	-	352	2,234	1.0	26.78	169.9	1.0
P_1	22	2,728	42	*	-	-	500	1,364	1.4	27.1	73.9	1.0
S_1	11.6	3,978	63	1.46	5,846	1	503	2,002	1.4	24.1	95.9	.88
P_2	26.4	1,195	19	1.34	1,601	.91	317	379	.9	60.2	71.9	2.25
S_2	6.6	2,145	34	.93	1,995	.64	463	993	1.3	10.5	22.6	.39
T	12.6	451	7.1	1.84	830	1.3	170	76.8	.48	92.2	41.6	3.4
B	1.4	4.4	.06	0	0	0	0	0	0	0	0	0
CV Brain	4.2			3.13	2	2	51.5	-	.10	0	0	0

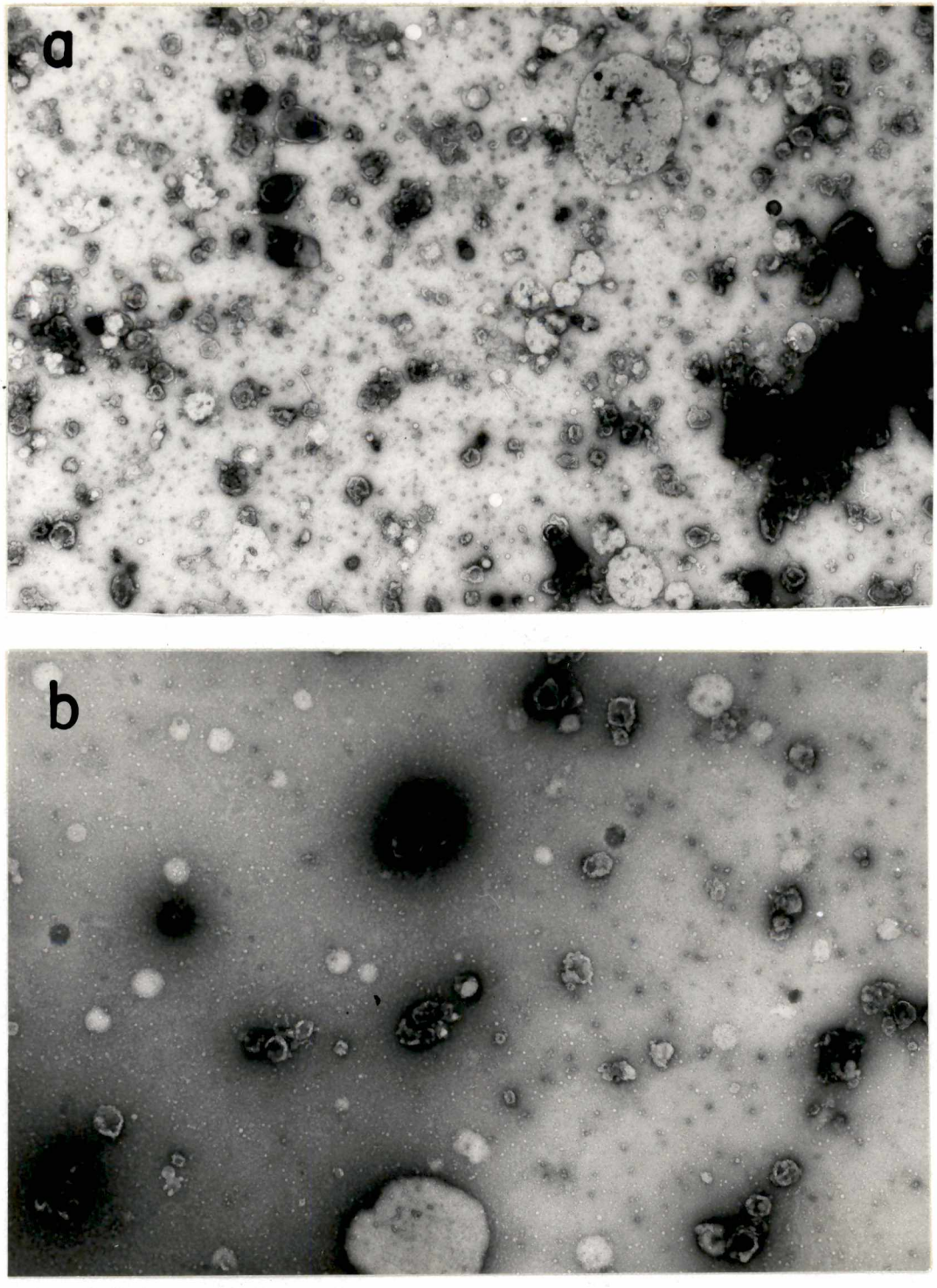

FIGURE 2. Electron micrographs of membrane fractions. **a**, negative stain of P_2 fraction, magnification 17,500x; **b**, negative stain of T fraction, magnification 20,400x.

few in number compared to the number of membrane vesicles.

In the second step of the isolation procedure the P_2 fraction was subjected to density gradient centrifugation to separate the lighter and more numerous membrane vesicles from the dense and sparse coated vesicles, Figure 1, page 51. Several factors were important for the successful employment of the gradient. First, $Ficoll_{70}$ (MW 70,000) was used for the gradient matrix instead of the more viscous $Ficoll_{400}$ (MW 400,000). Second, a temperature of at least 15°C was needed during the centrifugation run to reduce the viscosity of the gradient, and third, a short run time was required. At longer run times, greater than 6 hrs, membrane vesicle aggregates form, settle out and contaminate the coated vesicle pellet. Analysis of the fractions obtained from the density gradient with negative stain electron microscopy and for marker enzyme activities revealed that fraction T contained mostly membrane vesicles, Figure 2(b), that could be attributed to primarily NADH Cyto. C Red. activity (3.5 fold enrichment), but also Na^+-K^+ATPase activity (1.26 fold enrichment) and TPPase activity (.48 fold enrichment). The B fraction or pellet, Figure 3, representing only 0.06% of the total protein contained almost exclusively coated vesicles with no detectable activities for any marker enzymes tested.

Electron Microscopy

Further analysis of the B fraction with electron microscopy revealed that greater than 97% of all membrane profiles were coated vesicles with an average diameter of 75–85 nm. The outer surface of

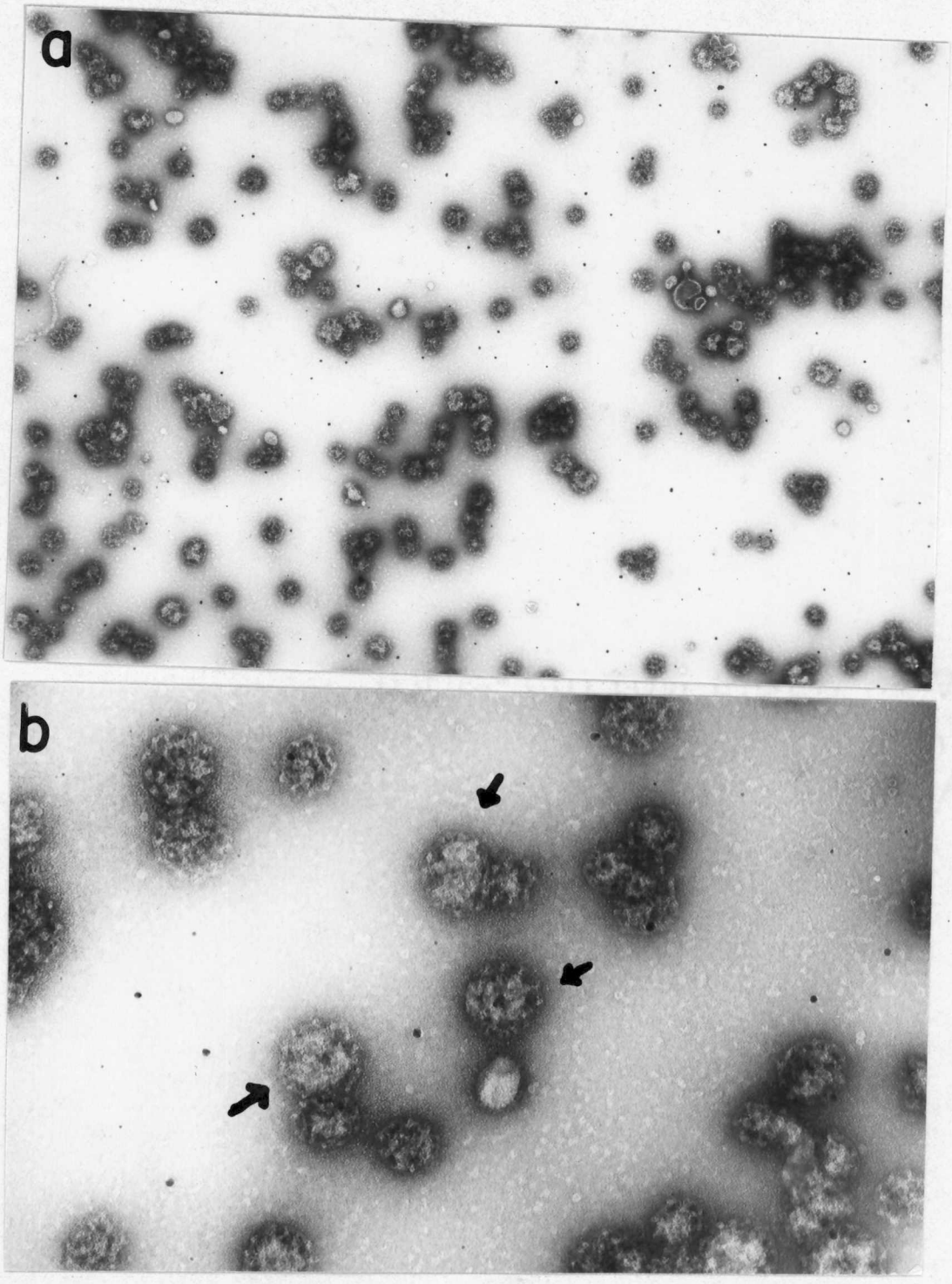

FIGURE 3. Electron micrographs of purified coated vesicles. **a**, magnification 35,000x; **b**, magnification 105,000x. Arrow coated vesicle with translucent core.

the coated vesicles when viewed with a negative stain exhibits the characteristic coat structure of hexagons and pentagons that are described in the literature (14, 18, 32, 34, 82). Approximately 75% of these vesicles contained an intact membrane (translucent core), arrow, Figure 3, whereas the remainding 25% appeared to be empty cages (no translucent core).

Protein Composition

SDS page electrophoresis performed on solubilized coated vesicle fractions revealed a banding pattern of proteins that was in agreement with other reports (14, 38, 54, 55, 67, 83). The predominant protein, identified as clathrin, bands at 180,000 MW and accounts for approximately 40-50% of all proteins. Other proteins were detected at 100,000 MW, 50,000 MW, and 32,000 MW positions in gel, Figure 4.

Recovery of Coated Vesicles

To determine the relative percent recovery of coated vesicles from the isolation procedure, coated vesicles derived from brain tissue by Pierces Method were labeled with ^{125}I and then were added to the corpus luteum homogenate at the beginning of the isolation procedure. Table 2 shows the recovery of ^{125}I-CV at the different steps in the isolation procedure. Nearly 38% of the total counts were detected in the P_1 fraction which represented lost ^{125}I-CV. Approximately 40% of the total counts of ^{125}I-CV were recovered in the P_2 fraction, the last differential centrifugation step. After the P_2 fraction was subjected to density gradient centrifugation only about

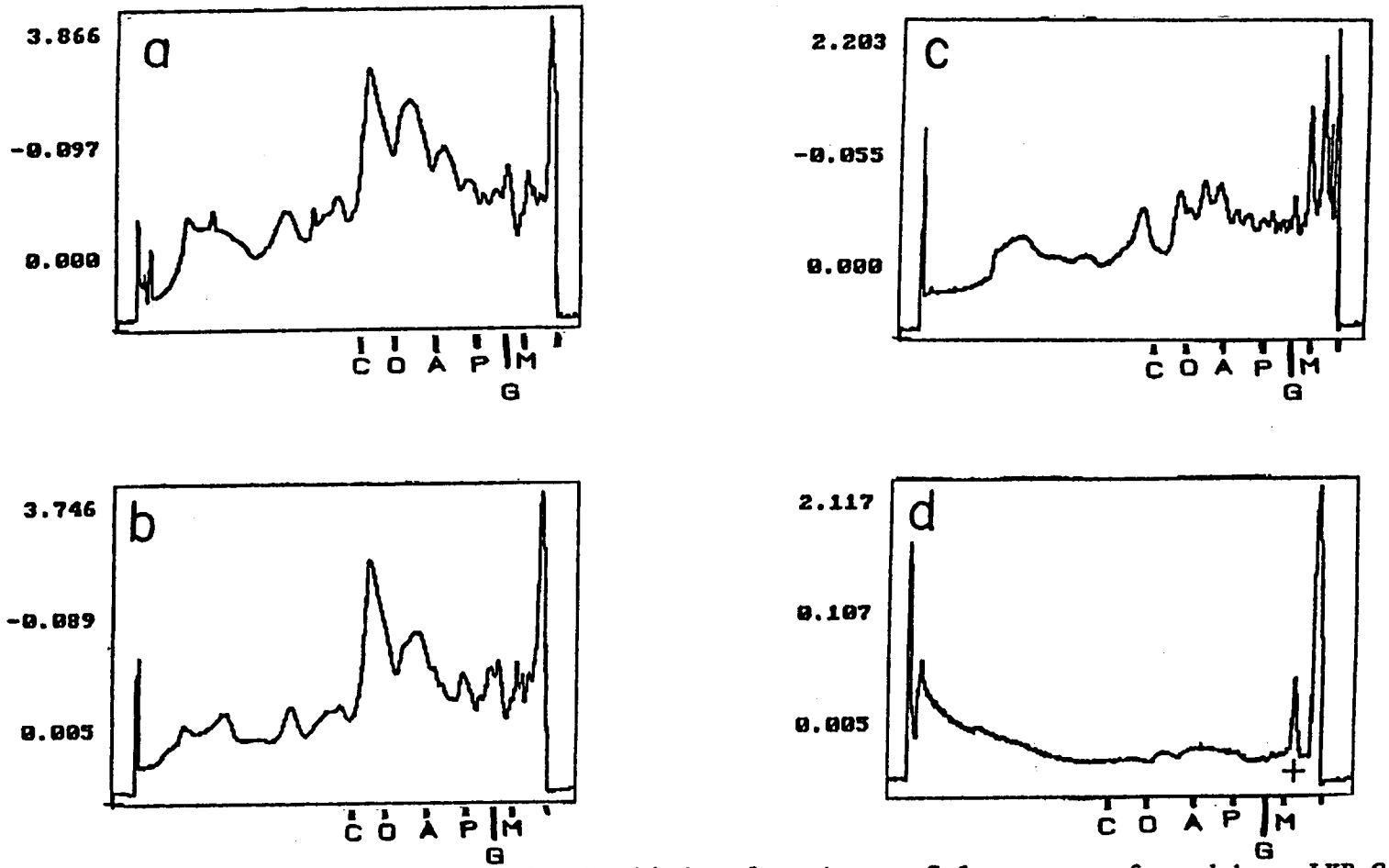

FIGURE 4. SDS gel electrophoresis of subcellular fractions. Gel scans performed by a LKB Gel Scan XL. Top of gels right hand side of scans. a, P_2 fraction; b, T fraction; c, coated vesicle fraction; d, isolated clathrin. Molecular weight markers: C, carbonic anhydrase - 29,000; Ov, Ovalbumin - 45,000; A, albumin - 66,000; P, phosphorylase B - 97,400; G, B-galactosidase - 116,000; M, myosin - 205,000.

TABLE 2. Recovery of ^{125}I-coated vesicles.

Fraction	Total Volume ml	Counts x 10^3 cpm/ml	Total Counts x 10^3 cpm/ml	Recovery % Yield
WH	60	113	6,780	100
P_1	22	116	2,552	38
S_1	73	54	3,942	58
P_2	16	174	2,784	41
S_2	65	13	846	12
T	30	27	833	12
B	2	960	1,920	28

28% of the ^{125}I-coated vesicles added to the homogenate at the beginning of the isolation procedure was recovered in the purified coated vesicle fraction, B.

IV. DISCUSSION

The data indicates that coated vesicles were isolated from the corpus luteum to near homogeneity. When the coated vesicle fraction was examined with electron microscopy, greater than 97% of all membrane profiles observed could be identified as coated vesicles. A survey of the activities of marker enzymes in this fraction revealed that there appears to be no contamination with the three most likely organelles: plasma membrane, Golgi membranes or endoplasmic reticulum. The morphology of coated vesicles, when viewed in the electron microscope with either negative stain or thin sections, appears identical with the structure of coated vesicles isolated from other tissues, i.e., adrenal cortex (48), adrenal medula (55), brain (10, 34, 54, 55, 82), liver (16, 37), B-Lymphocyte (41, 55), oocyte (56, 82), skeletal muscle (4), placenta (6, 52, 58). The outer surface of the coated vesicles is studded with a protein lattice work of hexagons and pentagons that are characteristic of the coat structure (14, 15, 18, 31, 32, 34, 36, 42, 56). Even the kinds of proteins present in this fraction and their relative amounts as determined by SDS polyacrylamide gel electrophoresis are in complete agreement with those reports on SDS PAGE for coated vesicles isolated from other tissues (6, 10, 15, 41, 48, 65, 82). The results from both

electron microscopy and marker enzyme assays correlate well with each other and provide evidence for reasonable homogeneity of this fraction and the efficiency of the isolation procedure employed.

The procedure used for isolating coated vesicles from the corpus luteum was well suited for this tissue type. Several problems inherent in the ultrastructure of the granulosa lutein cell, the principle cell in this tissue type, had to be overcome to successfully purify coated vesicles. The granulosa lutein cells of the corpus luteum possess many large, secondary lysosomes which are sensitive to both mechanical agitation and osmotic shock, and if ruptured, the released hydrolytic enzymes will degrade the coated vesicles. This cell type also contains within its cytoplasm many swirls, sheets and tubules of endoplasmic reticulum that fragment during homogenization and reseal to form membrane vesicles about the same size as coated vesicles. These membrane vesicles in the whole homogenate greatly outnumber coated vesicles and interfere severely with the isolation procedure. The procedure for isolating coated vesicles, outlined in this paper, eliminated the problem of enzymatic degradation of coated vesicles and circumvented the problem of separating the numerous membrane vesicles and their aggregates from coated vesicles. This procedure only required two differential centrifugation steps, one density gradient and could be completed within ten hours, unlike the more complex and longer 1-2 day procedures reported in the literature (6, 38, 49, 54, 58, 60).

The relative efficiency of any procedure used to isolate an

organelle can be measured by the yield and homogeneity of the final organelle product. On this basis, the procedure used for the isolation of coated vesicles, outlined in this paper, seems acceptable by present standards. The final coated vesicle fraction was nearly homogeneous and the yield appears to be greater than that achieved in other tissues. Although the final coated vesicle fraction contained only 0.06% of the total protein of the whole homogenate, the amount of ^{125}I-CV recovered from the starting homogenate was approximately 28%. This relatively high recovery (the reported average recovery from other systems was 5-10% (48, 65) was probably obtained only because few steps were required in the isolation procedure. The fewer the steps involved, the fewer the chances of loosing coated vesicles. Most of the coated vesicles that were lost during the isolation procedure were lost to the first differential centrifugation pellet, P_1. Approximately 40% of the ^{125}I-coated vesicles added to the homogenate at the start of the isolation procedure were located in this pellet. No attempts have yet been made to develop techniques to reduce this loss but the overall yield of the isolation procedure might be improved by washing (rehomogenizing-recentrifuging) the P_1 fraction repeatedly until a higher percentage of vesicles is recovered.

The isolation procedure also appears to be applicable to other tissue types. Coated vesicles were isolated from porcine brain using the same buffer and procedure as described in this paper. Approximately 95% of all membrane profiles observed in this fraction

with the electron microscope could be identified as coated vesicles. Analysis of this fraction by marker enzyme assays revealed that there was, however, a substantial amount of activity for Na^+-K^+ATPase, a plasma membrane marker. Whether this marker enzyme activity represents a natural constituent of brain coated vesicles or contamination from plasma membrane is not known. However, coated vesicles isolated from brain by other investigators using other procedures do not appear to have any substantial activity for plasma membrane marker enzymes (67) and therefore, the marker enzyme activity detected in this fraction probably represents a contamination from plasma membrane. Although the isolation procedure for coated vesicles was not so effective on brain tissue as with the corpus luteum, it was, however, efficient enough to result in a highly enriched fraction of coated vesicles from the brain in about a third the amount of time needed with other procedures. Modifications of this procedure, such as altering the density in the step gradient, may be necessary to obtain a more purified fraction of coated vesicles from the brain. The general applicability of this procedure for isolating coated vesicles from other tissues is not known, but probably will require subtle changes in the density of the step gradient, as is the case with brain tissue, to compensate for subtle differences in the size and density of coated vesicles and contaminating membrane vesicles.

Coated vesicles derived from other tissues have a wide range of sizes, Table 3. The range varies from 60 nm in diameter in brain tissue (54) to 150-250 nm in diameter in chick oocytes (56). Coated

TABLE 3. Comparison of coated vesicle diameters from different tissues. Explanation of abbreviations: in situ - NT, in vacinity of nerve terminal; in situ - PM, in vacinity of plasma membrane; in situ - Golgi, in vacinity of Golgi apparatus; ThnSx, thin sectioned; NgStn-UA, negative stained with uranylacetate; MD, mean diameter in nanometers; Mx, maximum diameter; R, range; (*) estimate based on measurements of coated vesicles in micrographs taken from cited reference.

TISSUE SOURCE	METHOD OF OBSERVATION	DIAMETER OF CV (nm)	REFERENCES
3T3-L, Adipocytes	In situ-PM, ThnSx	120 MD	17
Bovine Adrenal Cortex	isolated, NgStn-UA	60 MD, 85 MX	48
Bovine adrenal Medulla	isolated, NgStn-UA	55-90 R	55
Calf Brain	isolated, NgStn-UA	70 MD & 100-110 MD	10
Guinea Pig Brain	isolated, NgStn-UA	50-100 R	34
Pig Brain	isolated, NgStn-UA	60 MD	54
	isolated, NgStn-UA	75 MD, 60-100 R	82
Rat Post. Pituitary	in situ-NT, ThnSx	65 MD*	16
Rat Liver	isolated, NgStn-UA	85-145 R	37
	isolated, NgStn-UA	70 MD & 100-110 MD	10
	in situ-PM, ThnSx	100 MD	22
	in situ-Golgi, ThnSx	40-60 R	35
Human B Lymphocyte	isolated, NgStn-UA	60-90 R*	41
Lymphoma E1-4	isolated, NgStn-UA	100-120 R	55
Chicken Oocyte	isolated, NgStn-UA	150-250 R	56
	isolated, NgStn-UA	85 MD 60-105 R	82
Human Placenta	isolated, NgStn-UA	70-120 R*	52
	isolated, NgStn-UA	75-120 R	6
	isolated, NgStn-UA	50-100 R	58
Chick Embryo Skeletal Muscle	isolated, NgStn-UA	75 MD, 60-160 R*	4
Chick Myotube	in situ-PM, ThnSx	60-100 R	9
	-Golgi	40-60 R	
Rat Spermatid	in situ-PM, ThnSx	150-200 R	26
	-Golgi	70-80 R	
Rat Vas Deferens	in situ-PM, ThnSx	100 MD	21
	-Golgi	75 MD	

vesicles isolated from the corpus luteum appear to have an intermediate size with an approximate mean diameter of 75-80 nm. Even within the same cell type the size of coated vesicles may vary considerably (9,21, 26,37). Friend and Farquhar (21) were the first to describe that in epithelial cells there are two classes of coated vesicles based on size. Coated vesicles derived from the Golgi apparatus have a mean diameter of 75 nm whereas coated vesicles derived from the invagination of coated pits during endocytosis are larger and measure approximately 100 nm in diameter. A similar observation was made on granulosa lutein cells in which a smaller sized coated vesicles mean diameter 75-85 nm is associated with and derived from the Golgi apparatus and also a larger sized coated vesicle 110-120 nm in diameter is derived from invaginations of coated pits during endocytosis, see Figure 5. Based on this one criterion of size, the coated vesicles isolated from the corpus luteum using the procedure outlined in this study most likely were derived from the Golgi apparatus.

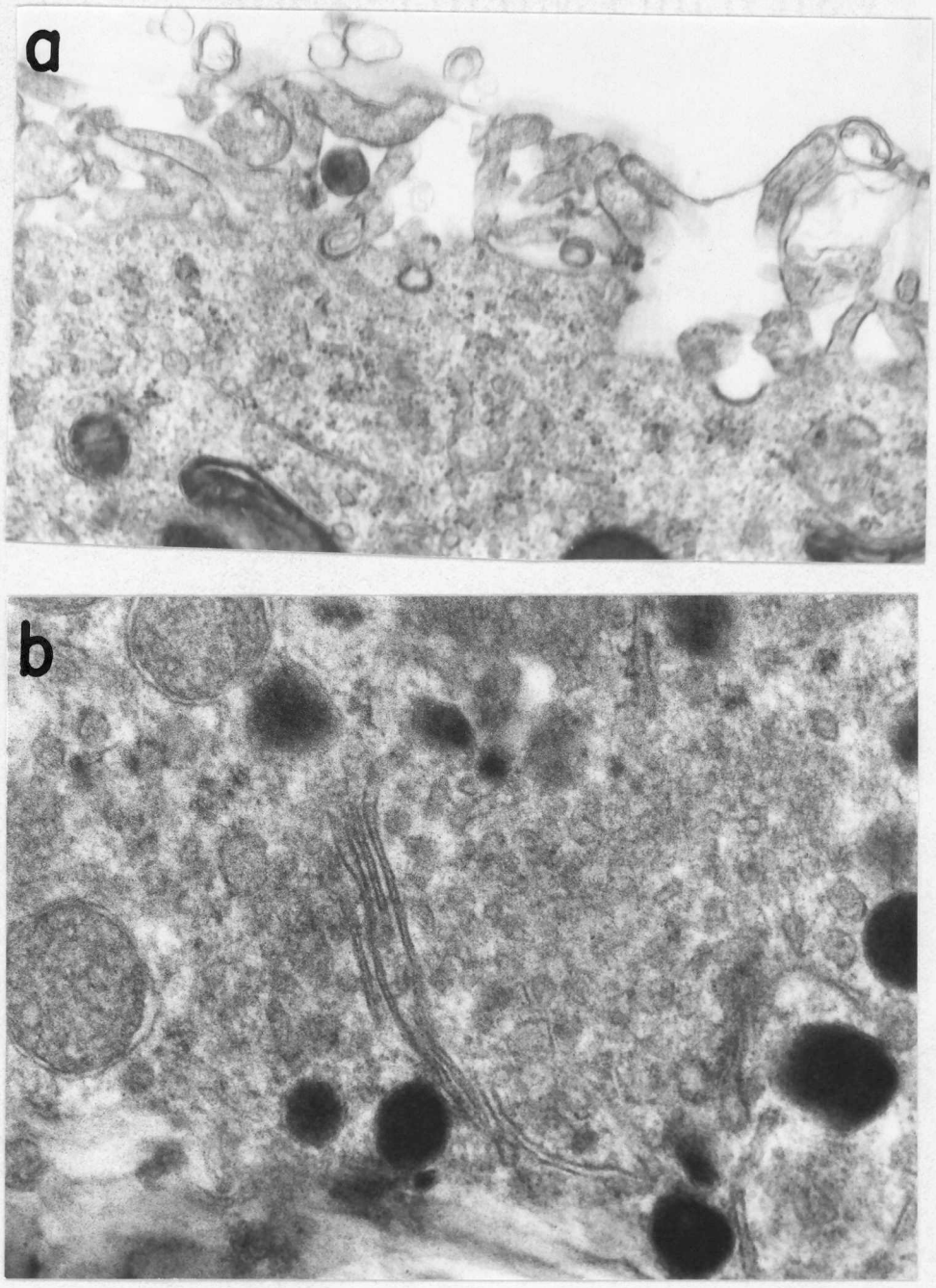

FIGURE 5. Electron micrographs of granulosa lutein cell. **a**, shows coated pits and coated vesicles on plasma membrane. Magnification 61,600x. **b**, shows Golgi apparatus and associated coated vesicles. Magnification 70,400x.

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PART 3

GONADOTROPIN AND LDL RECEPTORS ARE PRESENT WITHIN COATED VESICLES ISOLATED FROM THE CORPUS LUTEUM OF THE PIG

I. INTRODUCTION

The granulosa lutein cell of the corpus luteum possesses receptors for both luteinizing hormone (LH) (1, 12, 14, 15, 24, 26, 33, 36, 38, 49, 54, 68, 71, 74, 86, 88, 97, 99, 106, 128, 129, 134, 135) and low density lipoprotein (LDL) (8, 16, 17, 27, 39, 70, 83, 96, 117, 119, 136, 154, 165, 166) on its outer surface. The distributions of these two receptors over the cell surface appears to be similar. The gonadotropin receptors which bind LH, the major secretagogue of this cell type, are spread out over its microvillus border in a diffuse pattern (5, 6, 7, 12, 25a, 100). The LDL receptors which are involved in adsorption of LDL, a major source of cholesterol for steroid synthesis, also appear to be distributed in a diffuse pattern over the cell surface of this cell type with less than 4% initially concentrated in coated pits (119). In contrast, approximately 50 - 80% of LDL receptors on fibroblast are located in coated pits (9, 10, 11, 118, 141a).

Both types of receptors appear to be actively internalized by the cell through a similar pathway, although the exact fate of each receptor is not known. In fibroblasts the LDL receptors, because of their location in coated pits, are efficiently internalized by the cell on a continuous basis even though they may not be bound with LDL (9, 26a, 27a, 28, 64, 65, 118). After internalization of the LDL-receptor complex, LDL dissociates from its receptor in an acidic compartment, the endosome. The free LDL is then channeled to lysosomes for degradation, whereas most of the receptors are recycled

to the cell surface. However, eventually even the LDL receptors are destroyed in lysosomes. In fibroblast, the internalized LDL receptors are estimated to be recycled to the cell surface within a 12 min. period of time and each receptor may be reused up to 150 times in its 30 hr. lifespan (27a, 28, 64). In the granulosa lutein cell, LDL appears to be internalized through a similar pathway. Although the exact fate of the LDL receptor in granulosa cells has not been fully characterized, receptor recycling may also operate because this cell type, like other steroid producing cells, relies heavily on lipoproteins as the predominant source of cholesterol (8, 16, 17, 70, 160).

The gonadotropin receptors on the surface of lutein cells once bound with LH or hCG form small aggregates. These complexes appear to move slowly to coated pits (1, 4, 5, 6, 7, 12) where they are internalized by endocytosis (1, 4, 12, 41, 43, 47, 65, 72, 109, 113, 128, 129, 139) in what appears to be the same coated pit/coated vesicle pathway as used by the LDL receptor. However, once internalized, LH probably does not dissociate from its receptor in the endosome compartment, as is the case for LDL in fibroblast, and the entire hormone-receptor complex is channeled to secondary lysosomes for degradation (13). The half life of the LH receptor is approximately 8-24 hrs (1, 38, 41, 104, 111).

In the granulosa lutein cells, if the internalization and subsequent destruction of these receptors were to continue even at these slow rates, eventually the cell would loose its ability to

respond to new challenges of ligand. This is not, however, what is observed. In the case of LH receptors, apart from the initial down regulation (12, 42, 75, 84, 139, 141, 155, 161, 179), the cell regains its ability to respond to new challenges of LH within several hours (51, 75, 109, 132, 161). Therefore, some mechanism must operate in these cells to replace those receptors lost from the cell surface (1, 95). The primary objective of this paper was to analyze the most plausible mechanism responsible for this replacement process.

The replacement of most glycoproteins on the plasma membrane (PM) is thought to occur according to the mechanism as described by Rothman and Lodish (145) for the insertion of viral glycoproteins into the plasma membrane of the host cells. New proteins are incorporated into the membrane at the rough endoplasmic reticulum (RER) compartment where they are modified by the addition of core sugars rich in mannose. From here, the glycoproteins are then transferred to the Golgi compartment where further modification of the sugars occurs followed by selective packaging of these glycoproteins into membrane vehicles for transport and insertion into the plasma membrane. The synthesis of new LDL and LH receptors most likely will follow this mechanism since both of these receptors are membrane-bound glycoproteins. In the granulosa lutein cells, earlier studies have indicated that there is a large intracellular pool of unbound LH receptors (7). Many of these receptors have been localized in situ over the Golgi apparatus and also in isolated Golgi enriched fractions using ^{125}I-labelled human chorionic gonadotropic hormone (hCG) (110,

137, 138). In fibroblasts, the synthesis of the LDL receptors has been well characterized (48, 162) and the Golgi apparatus appears to be a locus of intracellular receptors. Presumably, these intracellular receptors for LH and/or LDL, particularly those associated with the Golgi apparatus, represent newly synthesized receptors or recycled receptors in transit to the PM. However, the actual mechanism of transport of these presumed to be newly synthesized receptors to the PM is uncertain especially in our cell type.

In the granulosa lutein cell, secretory granules and coated vesicles are the only known membrane bound structures that have been observed to be directly derived from the Golgi apparatus that later insert into the PM making them the two likely candidates for receptor transport. In earlier studies by our laboratory, it was shown that secretory granules isolated from our cell type do not possess any unbound LH receptors, and therefore, could not be involved in LH receptor transport. Our new hypothesis is that coated vesicles are the actual vehicle for transport of LDL and/or LH receptors from the Golgi apparatus to the PM.

Coated vesicles first described by Roth and Porter (144) have been found in every eukaryotic cell studied to date. They have been implicated in many cellular processes involving membrane surfaces: receptor-mediated endocytosis (9, 10, 33, 56, 57, 62, 63, 65, 67, 73, 78, 92, 98, 105, 114, 116, 120, 124, 130, 131, 143, 144, 149, 158, 159, 167, 168, 170–173); membrane recycling (52, 65, 79, 80, 124, 143,

159); intracellular protein translocation (59, 69, 80, 116, 124, 143, 144, 146, 147); and in exocytosis of both membrane and secretory proteins (20, 30, 34, 57, 58, 59, 89, 93, 116, 146, 147).

Coated vesicles as described by Kanaseki and Kodata (1969) (87) consist of a membrane vesicle surrounded by an icosohedral lattice work of protein. Pierce (1979) (45, 91, 121, 122) showed that the characteristic coat structure is made up principally of one protein called clathrin. The basic assembly unit for the coat structure appears to be a larger multimeric protein made up of three molecules each of clathrin and 100 K dalton protein that are attached together to a central locus to form a three armed structure, the triskelion (94, 163, 164).

In the formation of coated vesicles, triskelion are thought to first assemble together to form larger pentagon and hexagon structures in the membrane (22, 125). This forms coated patches. As more clathrin pentagons and hexagons assemble and move in place, coated pits form. With further addition of pentagons and hexagons, structural constraints of the triskelion assemblies are thought to force the underlying membrane to pinch off forming a coated vesicle. There is some question as to whether or not coated vesicles are completely detached from the membrane (120, 170, 171, 172), however, recent evidence indicates that coated vesicles are bonafide structures (56, 127, 164a).

The idea that coated vesicles may transport receptors between

membrane compartments is not new. Coated vesicles isolated from other tissues have been shown to possess a variety of receptors: liver--asialyoglycoproteins (62, 63) and mannose 6-phosphate (31, 32); muscle---acetylcholine (20); placenta--transferrin (22, 125) and immunoglobulins (125); adrenal cortex--LDL (108).

In this paper, to test the primary hypothesis on the mechanism in which receptors lost from the cell surface are replaced, isolated coated vesicles were examined for their content of LDL and LH receptors.

II. MATERIALS AND METHODS

Materials

All chemicals used in this study were purchased from Sigma Chem. Co., St. Louis, Mo., except for lactoperoxidase Grade B which was purchased from Calbiochem. Behring Corp., La Jolla, CA. Sodium Iodide-125, carrier free, was purchased from ICN Biomedicals Inc., Irvine, CA. Highly purified hCG (CR-121) prepared by Dr. R. Canfield [biological activity 13,450 IU/mg] was provided by the Center for Population Research, NICHD. All tissues used for experimentation were obtained from Lay's Meat Packing Co., Knoxville, TN.

Preparation of Crude Membranes

The procedure used for the preparation of crude membranes from the porcine corpus luteum is summarized in Figures 1 and 2. All steps in the isolation procedure were performed at 4°C. Pig ovaries were collected from the slaughterhouse in ice cold saline solution; and

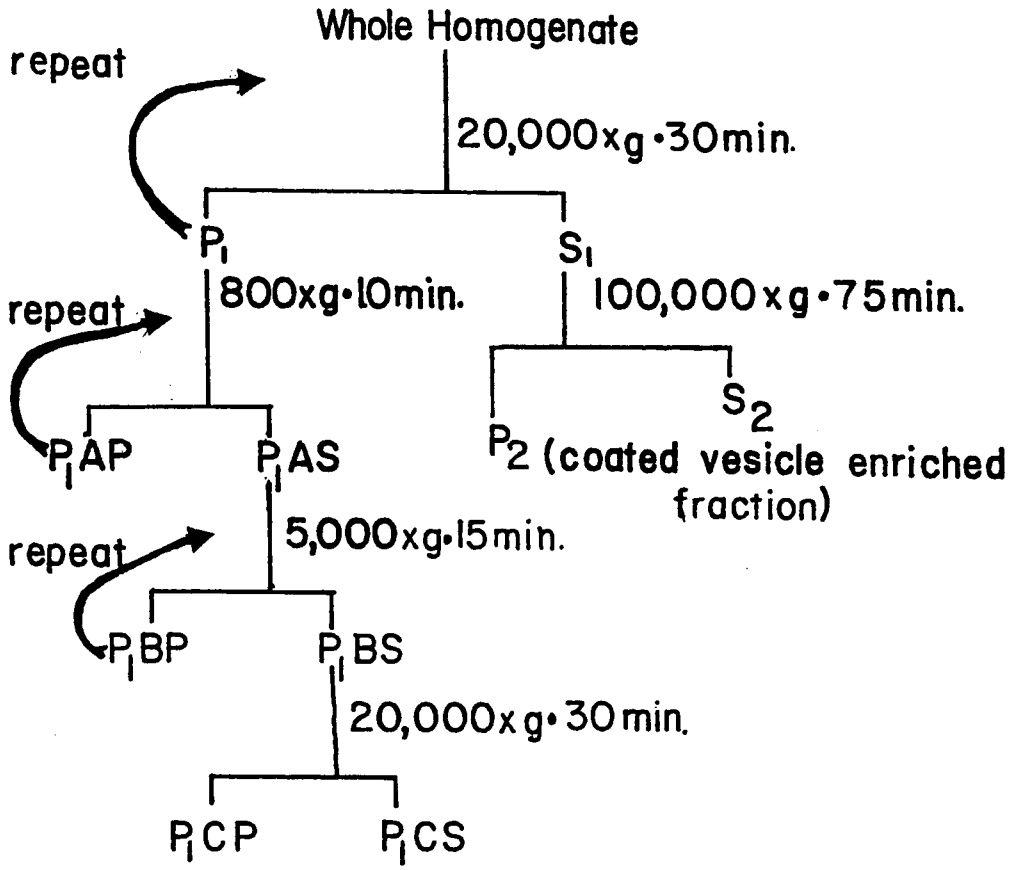

FIGURE 1. Flow diagram of differential centrifugation.

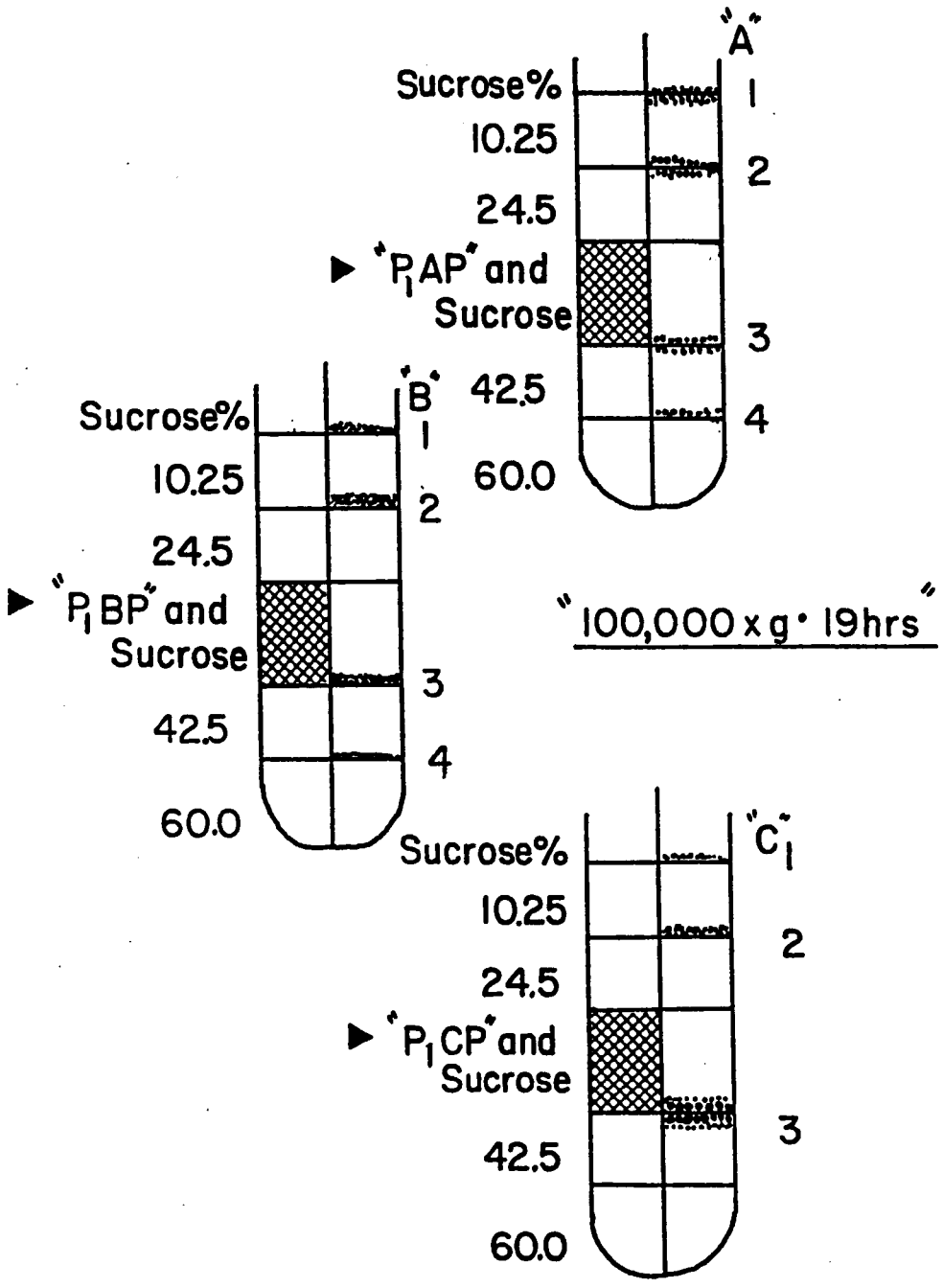

FIGURE 2. Density gradient centrifugation. Sample distribution: before centrifugation, left side of tube; after centrifugation, right side of tube.

brought back to the laboratory for further processing. Corpora lutea were excised from the ovaries and any extraneous connective tissue was removed. The tissue was minced using a razor blade and placed in buffer A (10 mM HEPES, pH 7.0, 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$, 0.02% NaN_3, 0.1 mM PMSF, 40 ug/ml soybean trypsin inhibitor, and 20 ug/ml TLCK). The volume was adjusted with buffer A to 30% w/v. Typically, 10-100 gm of tissue were used. Homogenization was accomplished using a 40 ml Dounce tissue homogenizer. Tissue was first ground using 6 strokes of the loose fitting pestle. The homogenate was then filtered through 3 layers of gauze to remove connective tissue sheets, followed by further homogenization using 15 strokes of the tight fitting pestle. The homogenate was centrifuged at 20,000xg for 30 min in a Beckman J-21C preparative centrifuge fitted with a JA-20 rotor. The supernatant (S_1) was poured off and saved. The pellets (P_1) were rehomogenized in fresh buffer A (half of original volume) and recentrifuged. The resulting supernatant S_1* was combined with S_1. The total supernatant was then centrifuged at 100,000xg for 75 min in a SW27 rotor with the Beckman L5-50 ultracentrifuge. The 100,000xg pellets (P_2) containing coated vesicles were pooled and further processed for isolation of coated vesicles as described elsewhere.

The crude membrane fraction (P_1) was further processed by differential and density gradient centrifugation to obtain enriched fractions of several organelles. (See Figures 1 and 2). The P_1 fraction was rehomogenized into half of the volume of the original

buffer with a Dounce homogenizer as described earlier. The P_1 fraction was then subjected to differential centrifugation involving three separate steps: A, 800xg for 10 min; B, 5,000xg for 15 min; and C, 20,000xg for 30 min. At each step the resulting pellet was rehomogenized in buffer and the centrifugation step was repeated. Supernatants were combined before beginning the next step. The three pellets resulting from the three steps (A, AP; B, BP; C, CP) were rehomogenized in buffer B (10 mM HEPES, pH 7.0, 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$) and each was subjected to density gradient centrifugation. All sucrose density solution used in density gradients were prepared in HEPES buffer B and all steps in the procedure were performed at 4°C. In preparation of samples, 4 ml of each sample was mixed with 6 ml of 51% w/v sucrose in buffer B (refractive index 1.4083). Each sample-sucrose mixture (10 ml) was placed in a SW27 cellulose nitrate tube, underlayed with two 7 ml sucrose solutions of greater density (60%, rf. ind. 1.4205 and 42.5%, rf. ind. 1.39605), and then overlayed with two 7 ml sucrose solutions of lighter density (24.5%, rf. ind. 1.3708 and 10.25%, rf. ind. 1.3507). The step gradients were centrifuged at 100,000xg for 19 hrs in a Beckman SW27 rotor. (See Figure 2.) After centrifugation, membrane fractions banding at interfaces between gradient steps were removed using a syringe and 16 gauge needle with bent bevel, diluted at least 5 fold with buffer B, and centrifuged at 100,000xg for 75 min in a Beckman SW27 rotor. The contents of each fraction were

characterized by measuring the activity of several marker enzymes as described in Part 2.

Isolation of Coated Vesicles

Coated vesicles were isolated from both porcine corpora lutea and porcine brain in the same way using fresh tissue obtained from the local slaughter house according to the methods described earlier.

Enzyme Assays

Activities for marker enzymes (Na^+-K^+)ATPase (EC 3.6.1.3), thiamine pyrophosphatase (EC 3.6.1.1) and NADH cytochrome C Reductase (EC 1.6.99.33) were measured with all appropriate controls with a few modifications of the methods outlined by Bramley and Ryan (25) and Wallach and Kamat (169) as described in Part 2. Enzyme assays were performed as three point fixed time assays except for NADH Cyto. C Red, which was a continuous time assay. In either case only the linear reaction rates were used to calculate enzyme activities. The activities were expressed as the quantity of product (nanomoles) formed per min per mg of protein. Protein was estimated by the method of Lowry (103) using bovine serum albumin as a standard.

Radioiodination of hCG

^{125}I-hCG was radioiodinated to a specific activity of approximately 35-40 uCi/ug using a modification of the lactoperoxidase procedure as described by Morrison and Bayse (112). In brief: To a polypropylene microfuge tube on ice the following solutions at 4°C were quickly added in order: 25 ul 0.5 M PO_4 pH 7.5, 10 ul hCG-CR-121

(1 mg/ml 0.05 M PO_4 pH 7.5), 20 ul $Na^{125}I$ (100 mCi/ml 0.01 N NaOH), 10 ul lactoperoxidase (0.1 mg/ml 0.05 M PO_4) and 10 ul H_2O_2 (40 ng), mixed by thumping and then incubated at 4°C for 4 min. The reaction was terminated by adding 100 ul of transfer solution (16% w/v sucrose, 0.05M PO_4, pH 7.5, 0.1% w/v gelatin) and mixing and then quickly transferring this to the top of a 5 ml sephadex G-25 (coarse) column that was previously flushed with column buffer (0.05 M PO_4 pH 7.5, 0.1% gelatin and 0.1 mg/ml soybean trypsin inhibitor). The reaction chamber was rinsed with 200 ul of transfer solution and this was added to the column. The separation of free ^{125}I from bound ^{125}I was accomplished by running the column with column buffer and collecting one ml aliquots that drained from below. The ^{125}I hCG peak and free ^{125}I peak were determined using a Beckman gamma 4000 counter.

Specific Binding ^{125}I-hCG

Binding of ^{125}I-hCG to various subcellular fractions was determined by a modification of the method of Roa (137). A 400 ul assay system was used containing 0.01 M PO_4, pH 7.4, 0.15 M NaCl, 0.1% BSA, 0.02% NaN_3, 1 mM EGTA, 0.5 mM $MgCl_2$, 5 mM octylglucoside, 0.01% saponin, 200,000 cpm ^{125}I-hCG and 50 ul of membrane fractions. The assay tubes were incubated at room temperature for 24 hrs in the presence and absence of 100 fold excess of unlabelled hCG (Sigma CG-B).

Separation of receptor bound ^{125}I-hCG from free ^{125}I-hCG was performed by a modification of the double precipitation with

polyethylene glycol method of Dufau and Charreau (53). In brief: The volume of the assay system was adjusted to 850 ul with the addition of PBS/BSA (0.01 M PO_4, pH 7.4, 0.15 M NaCl and 0.1% w/v Bovine serum albumin). To precipitate the bound hormone, 200 ul of 0.5% w/v rabbit IgG in PBS and 650 ul of 30% w/v polyethyleneglycol MW 6,000-8,000 (PEG) were added, the tubes were vortexed for 15 seconds and incubated at 0°C for 10 min. After which, the tubes were centrifuged at 800xg for 10 min in a Beckman TJ-6R table top centrifuge. The supernatants were removed by aspiration and discarded. The pellets containing precipitated bound hormone were resuspended in 900 ul of 0.2% triton X100 in PBS by vortexing followed by incubation at 0°C for 10 min. The bound hormone was reprecipitated by adding 650 ul of 30% w/v PEG vortexing and incubating at 0°C for 10 min. The tubes were then recentrifuged at 1500xg for 15 min. The resulting supernatants were removed by aspiration and discarded. The pellets were counted in a Beckman gamma 4000 counter. The specific binding was determined by subtracting the nonspecific binding (measured in the presence of 100 fold excess of unlabelled hCG) from the total.

Isolation of Porcine Low Density Lipoprotein

Porcine LDL (density 1.020-1.067 g/ml) was prepared from plasma by ultracentrification by a modification of the method of Havel (77). In brief: One to two liters of porcine blood were collected in .1% Na_2EDTA and 0.02% NaN_3 on ice. The blood was centrfuged at 1000xg for 15 min in a Beckman JA-20 rotor to remove blood cells. The plasma was removed and its density was adjusted to 1.020 g/ml with KBr (X ml

plasma X0.018 gm KBr/ml) and then centrifuged at 200,000xg for 25 hrs. at 4°C in a Beckman 60Ti rotor. After centrifugation, the middle yellow layer between the upper clear and lower red layers was removed. This layer contained both LDL and HDL. The density of the LDL-HDL fraction was adjusted to 1.067 with KBR (X ml of fraction X0.075 gm KBr/ml) and then centrifuged at 200,000xg for 25 hrs. at 4°C in a Beckman 75Ti rotor. After centrifugation, the very top yellow layer containing LDL was removed. The LDL fraction was then dialyzed extensively against a 10 mM HEPES buffer, pH 7.5, containing 0.15 M NaCl and 0.3 mM Na_2EDTA, sterilized by membrane filtration, and stored at 4°C. Purified LDL was used within three weeks of preparation. The homogeneity of the LDL fraction was determined by agarose gel electrophoresis and observation by negative stain electron microscopy.

Electrophoresis of LDL

Horizontal agarose gel electrophoresis was performed on precoated 10 x 12.5 cm glass plates according to the method of Noble (115). The plates were coated with a 2 mm thick layer of 0.4% agarose–0.12% agar containing 2% bovine serum albumin and 0.05 M sodium barbital buffer, pH 8.6. After the gel cooled, four troughs (2 x 10 mm) were cut in the gel. Samples were mixed with an equal volume of warmed 1% agarose in 0.05 M barbital buffer and poured into the troughs. After the samples were cooled, the plates were placed in a Biorad horizontal electrophoresis unit, model 600, connected to buffer chambers containing 0.05 M sodium barbital buffer, pH 8.6, and subjected to

electrophoresis with a constant current of 30 mAmp for 3 hrs. at room temperature. Afterwards, the plates were fixed 1 hr. in 5% acetic acid-75% EtoH, stained 5 hrs. in a diluted solution of Sudan Black B in 60% EtoH (1 mg/ml).

Radioiodination of LDL

LDL was iodinated by the method of Macfarlane using iodine monochloride as modified by Bilheimer et al (21). In brief: To a 1.5 ml polypropylene microfuge tube the following solutions at 4°C were added in order: 250 ul of freshly prepared LDL (14.0 mg/ml), 250 ul 2 M glycine, pH 10.0, 30 ul of NaI^{125} (100 mCi/ml) and 50 ul of freshly diluted iodine monochloride (from a new bottle) solution (0.2 mg/ml in 2 M NaCl). The contents were briefly mixed and then extensively dialyzed against 10 mM HEPES buffer, pH 7.5, containing 0.15 M NaCl and 0.3 mM Na_2EDTA. Specific activity was approximately 360 cpm/ng of LDL protein. Any dilutions of ^{125}I-LDL used for binding studies were made with HEPES buffer containing bovine serum albumin (20 mg/ml).

Binding of ^{125}I-LDL to Membrane Fractions

The binding of ^{125}I-LDL to membrane fractions was determined by a modification of the procedure outlined by Basu (18). The standard binding assay was performed at 4°C in polyethylene 12 x 75 mm tubes with a 300 ul total volume of 25 mM HEPES buffer, pH 7.5 containing 0.15 mM NaCl, 0.1 mM EDTA, 1 mM $CaCl_2$, 0.02% NaN_3, 20 mg/ml BSA, 5 mM octylglucoside, .01% saponin, 25-100 ug of membrane fraction, and 400,000 cpm ^{125}I-LDL (360 cpm/ng of LDL protein) either alone (total

binding) or in the presence of excess unlabelled LDL (1 mg/ml) (nonspecific binding). At the end of 6 hours, 3.5 ml of HEPES buffer with BSA (20 mg/ml) were added to samples and the tubes were centrifuged at 8,000 xg for 45 minutes at 4 ^{o}C in a Sorval HS-4 rotor with 12 x 75 mm tube carriers. The free radioactivity (supernatant) was separated from bound radioactivity (pellet) by aspiration. Pellets were counted in a Beckman gamma 4000 counter. The specific binding was determined by subtracting the nonspecific binding from total binding.

Preparation of Coated Vesicles for Binding Studies

To determine whether or not LH and/or LDL receptors associated with coated vesicles are localized on the inside or outside, the vesicles were first treated with proteases in the following manner before they were subjected to binding with either ^{125}I-hCG or ^{125}I-LDL. Coated vesicles derived from the corpus luteum were suspended in buffer B (10 mM HEPES, pH 7.0, 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MnCl_2$, and 0.02% NaN_3) 1 mg of protein per ml. The suspension was aliquoted into three groups and placed in SW60 polyallomer centrifuge tubes; group A, 200 ul; group B, 400 ul; group C, 400 ul. To group A, 20 ul of detergent (50 mM octylglucoside and 0.1% saponin in dH_2O) was added along with 12 ul of enzyme solution (2 mg/ml trypsin (11,000 B.I.U./mg of protein) and 1 mg/ml chymotrypsin). To group B only 21 ul of the enzyme solution was added. Nothing was added to group C. All three groups were then incubated at 37^{o}C for 45 min. The digestion was terminated by diluting each tube to 4.0 ml with ice cold buffer A

containing 4 ug/ml soybean trypsin inhibitor, 0.1 mM PMSF and 0.5 mg/ml of BSA followed by centrifugation at 100,000xg for 45 min. in a SW60 rotor at 4°C. Supernatants were removed by aspiration and the pellets were resuspended in buffer A; group A, 200 ul; group B, 400 ul; group C, 400 ul. All three groups were divided into equal aliquots for binding studies; one half was saved for binding with ^{125}I-hCG and the other half was used for binding with ^{125}I-LDL.

Binding with ^{125}I-hCG

The processed coated vesicles for each of the three groups (A-C) were subjected to binding with ^{125}I-hCG as described above except that the assay system for half of samples from groups B and C did not contain detergent.

Binding with ^{125}I-LDL

The procedure used for binding ^{125}I-LDL to coated vesicles was a modification of the basic procedure outlined above for binding with membranes. All buffers and solutions used were adjusted to a pH 7.0 and the binding was performed in the absence of detergents, although in half of each group A-C, the coated vesicles were treated with detergents and washed prior to use. Detergent treated coated vesicles were prepared by incubating them in the presence of 25 mM octylglucoside and 0.05% saponin on ice for 20 min, after which, the detergent was diluted 20 fold with buffer and centrifuged at 100,000xg for 45 min. in a SW60 rotor. The supernatants were decanted, the

pellets were resuspended in buffer and then were used in the binding study as described above except for modifications noted. The bound ^{125}I-LDL was separated from the free ^{125}I-LDL by centrifugation at 100,000xg for 45 min. in Beckman SW60 rotor. Supernatants were removed by aspiration and the pellets were counted in a Beckman gamma 4000 counter.

Electron Microscopy

Thin sections: For tissue; thick sections were cut through the middle of corpora lutea. The tissue was fixed for 1 hr in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C, after which the tissue was cut into smaller pieces and put in fresh fixative overnight at 4C. Tissue was rinsed in 5% sucrose in 0.1 M sodium cacodylate buffer, post-fixed for 1 hr in 1% OsO_4 in 0.1 M cacodylate buffer, stained en bloc for 2 hours in 1% uranylacetate, dehydrated in ethanol alcohol and embedded in epon. Thin sections of samples cut with a diamond knife on a Riechert ultramicrotome, were stained with uranyl acetate and lead citrate and viewed in a Hitachi H600 electron microscope.

For membrane fractions; small pieces of pelleted subcellular membrane fractions were removed with a spatula, fixed and stained with the same protocol used for the tissues except that an extra step was added. Immediately after osmication, the membrane fraction pellets were incubated for 15 min in 0.25% tannic acid in 0.05 M cacodylate buffer and rinsed in 0.1 M cacodylate buffer before proceeding with

the next steps.

Negative staining: For negative staining, membrane fractions diluted with buffer B (approx. 0.5 mg/ml) or diluted LDL fractions were deposited on formvar, carbon-coated and glow discharged copper grids for 1 min, excess samples were removed and the grids were washed with several drops of 2% uranyl acetate in distilled H_2O. The grids were then stained for 30 sec with the uranyl acetate solution after which the excess was removed by drawing it off with filter paper and the grids were then allowed to air dry. Grids were viewed in the Hitachi H600 or H800 at 100K volts.

III. RESULTS

Isolation of Membrane Fractions

Membrane fractions of either plasma membrane, Golgi apparatus or endoplasmic reticulum were isolated from the corpus luteum to be used in competitive binding assays for both ^{125}I-hCG and ^{125}I-LDL. These membrane organelles are known loci for both the hCG and LDL receptors and were therefore used as reference membrane for comparative ^{125}I-hCG and ^{125}I-LDL binding assays with isolated coated vesicles. The procedure used in their preparation involved both differential and density gradient centrifugation steps. A summary is illustrated in Figures 1 and 2, pages 88 and 89. The purpose of the differential centrifugation was to partially separate membrane components from each other according to their sedimentation coefficients and also to concentrate the membranes into smaller volumes. Table 1 shows

TABLE 1. Marker enzyme activities and binding of ^{125}I-hCG and ^{125}I-LDL to subcellular fractions. SA, specific activity (enzyme activity [nmoles of product/min] divided by mg of protein or binding of ^{125}I-ligand [cpm] divided by mg of protein).

Fraction	Volume	Protein			Total Phosphatase		$(Na^+ - K^+)$ ATPase		Thiamine Pyrophosphatase		NADH Cytochrome C. Reductase		Binding of ^{125}I-hCG		Binding of ^{125}I-LDL	
													SAx10^3	Totalx10^6	SAx10^3	Totalx10^6
	ml	mg/ml	Total	% Yield	SA	Total	SA	Total	SA	Totalx10^3	SA	Totalx10^3				
WH	300	21	6,346	100	5.5	35,096			352	2,234	26.8	170	51.0	329.9	47.3	300
P_1	124	22	2,728	42	3.6	9,943			500	1,364	27.1	74	41.7	113.9	80.9	220.8
S_1	343	12	3,978	63	5.1	20,329	1.46	5,846	503	2,002	24.1	96	54.3	216.0	49.0	195.1
P_2	45.3	26	1,195	19	5.8	6,943	1.34	1,613	317	375	60.2	72	59.3	70.8	85.5	102.2
S_2	321.9	7	2,145	34	5.3	11,471	.93	1,995	463	993	10.5	23	8.1	17.5	23.9	51.3
P_1AP	44	24	1,064	17	2.4	1,805			315	241	18.4	20	43.7	46.5	69.0	73.4
P_1AS	134.3	11	1,504	24	4.0	6,087	.21	312	413	622	30.6	46	44.8	67.4	107.1	161.1
P_1BP	16.8	20	342	5	2.8	962			263	90	22.6	8	78.4	26.8	149.1	51.0
P_1BS	150.1	8	1,200	19	3.8	4,534	.09	116	539	647	35.1	42	44.4	53.2	94.8	113.7
P_1CP	30.9	11	352	5	6.6	2,332			1,041	367	52.4	18	127.4	44.9	231.9	81.6
P_1CS	135.2	4	595	9	3.3	1,990			563	336	42.5	25	5.2	3.1	70.1	41.7
A1	15.9	.7	11.1	.2	4.4	49	4.4	49.3	1,916	21	145.2	1.6	38.3	.42	1,060.7	11.8
A2	16.6	1.4	23.3	.4	7.2	168	7.19	167.7	1,678	39	176.1	4.1	225.8	5.3	1,420.0	33.1
A3	17.4	6.4	111.3	1.7	4.6	512	1.75	195	649	72	105.3	12	183.5	20.4	132.0	14.7
A4	26.3	8.8	231.2	3.6	3.6	824	1.36	315.5	350	81			102.5	23.7	175.4	40.5
B1	5.6	.3	1.7	.02	10.3	17			1,010	11	129.6	.22	155.4	.26	2,503.4	4.2
B2	7.4	1.4	10.4	.16	16.0	166	2.21	22.9	4,073	42	66.9	.69	386.9	4.0	1,755.8	18.2
B3	6.0	17.6	105.6	1.7	4.7	497	4.7	497.4	465	49	46.6	4.9	85.6	9.0	44.0	4.6
B4	7.4	2.1	15.5	.2	1.8	29	1.84	28.7	862	13	67.2	1.0	93.8	1.4	487.8	7.6
C1	11	.6	6.6	.1	5.2	34			2,238	15	77.9	.51	159.7	1.0	4,217.8	27.7
C2	12	5.7	68.3	1.1	9.5	650	9.5	649.7	1,659	113	56.4	7.5	238.2	16.3	414.9	28.3
C3	12.5	10.7	133.6	2.1	4.1	547	4.09	546.7	564	75	60.9	8.1	145.1	19.4	152.5	20.4
T1	12.2	.6	7.3	.1			0		0	0	169.5	1.2	143.0	1.0	2,266	16.6
T2	15.2	16.4	248.7	3.9	2.5	617	.29	72.9	360	89	69.7	17	44.8	11.1	128.8	32.0
T3	12.8	.5	6.4	.1	47	302	47	302.2	0	0	124.9	.79	90.9	.58	1,213.4	7.7
T	35.8	12.6	450.9	7.1	3.0	1,358	1.84	830	170	77	92.2	42	105.9	47.8	169.0	76.2
B	3.2	1.4	4.4	.06	0		0		0	0		0	78.7	.35	825.9	3.6
CV Brain	4.0	4.2			6.8		3.13		51			0	6.4		747.5	

activities of marker enzymes of all fractions obtained from the isolation procedure for the three types of membrane organelles that were concerned with: plasma membrane (PM), (Na^+-K^+)ATPase; Golgi membrane, thiamine pyrophosphatase; endoplasmic reticulum (ER), NADH cytochrome C reductase (NADH Cyto. C Red.). The P_1 fraction obtained from the first centrifugation step contained 42% of the total protein, 33% of the total phosphatase, 40% of TPPase and 44% of NADH cytochrome C reductase activities. Electron micrographs of this fraction (not shown) revealed presence of connective tissue sheets, unbroken cells, nuclei, mitochondria, lysosomes, secretory granules and large membrane vesicles. This fraction because of its rich membrane content, was further subfractionated using differential centrifugation into three pellets, AP, BP, and CP, illustrated in Figure 1, page 88. Fraction AP contained connective tissue sheets, unbroken cells, nuclei and some mitochondria. Fraction BP contained mitochondria, lysosomes, secretory granules and some large membrane vesicles. Fraction CP contained mostly membrane vesicles. The P_2 fraction was saved and used as the source of coated vesicles for their purification as described below. The marker enzyme activities are depicted in Table 1.

Since each of these fractions was not homogeneous in its type of membrane contents, further processing of the fractions using density gradients was required to obtain more pure membrane fractions. For each of the pellets subjected to density gradient centrifugation the resulting subfractions are illustrated in Figure 2, page 89.

Only four of all of these fractions from the gradients were considered enriched enough in one or in combination of particular marker enzyme(s) to be used as a representative of a membrane organelle(s). The B_2 fraction possessed the greatest enrichment of TPPase activity (enrichment factor (EF) 11.5) compared to any other fraction. Although it was also enriched with NADH Cyto. C Red. (EF 2.5) and (Na^+-k^+)ATPase (EF 1.5). This fraction was labelled Golgi membrane and ER. The C_2 fraction was a mixture of membrane components and contained activities for (Na^+-k^+)ATPase (EF 6.5), TPPase (EF 4.7) and NADH Cyto. C Red. (EF 2.1). The T_1 fraction elicited a six fold enrichment of NADH Cyto. C Red. activity with no detectable activities for any other marker enzyme measured. This fraction was appropriately labelled reticulum fraction. The T_3 fraction possessed the greatest enrichment of Na^+K^+ATPase activity (EF 3.2), no detectable TPPase activity, and a four-fold enrichment in NADH Cyto. C Red. This fraction was labelled PM & ER.

The marker enzyme activities of these four fractions along with the whole homogenate (WH) and the P_2 fraction are summarized in Table 2 and Figure 3. The marker enzyme activity for each fraction is expressed as an enrichment factor [specific activity of the fraction (nanomoles of product per min. per mg protein) divided by the specific activity of the whole homogenate].

Isolation of Coated Vesicles

The procedures for the isolation of coated vesicles from either the corpus luteum or brain were described previously. The coated

TABLE 2. Marker enzyme activities and ligand binding to selected fractions from Table 1. SA, Specific activity (enzyme activity [nmole product/min.] divided mg of protein or binding of ^{125}I-ligand [cpm] divided by mg of protein); EF, enrichment factor (specific activity of fraction divided by specific activity of whole homogenate).

	Total Phospatase		(Na^+-K^+) ATPase		TPPase		NADH Cyto.C.Red		Binding of ^{125}I-hCG		Binding of ^{125}I-LDL	
	SA	EF	SA	EF	SA	EF	SA	EF	SA x 10^3	EF	SA x 10^3	EF
WH	5.5	1	0	0	352	1	26.7	1	52.0	1	47.3	1
P_2	5.8	1.0	1.3	.92	317	.90	60.2	2.25	59.3	1.14	85.5	1.8
B_2	16.0	2.9	2.2	1.5	4073	11.6	66.9	2.5	386.9	7.44	1,755.8	37
C_2	9.5	1.7	9.5	6.5	1659	4.7	56.4	2.1	238.2	4.58	.414.9	8.78
T_1	0	0	0	0	0	0	169.5	6.3	143.0	2.75	2,266.2	47.9
T_3	47	8.5	47	32	0	0	124.9	4.7	91.0	1.75	1,213.4	25.7
B	0		0	0	0	0	0	0	78.7	1.51	825.9	17.4
CvBr	6.8		3.1	2.1	51.5	.15	0	0	6.4	.12	747.5	15.8

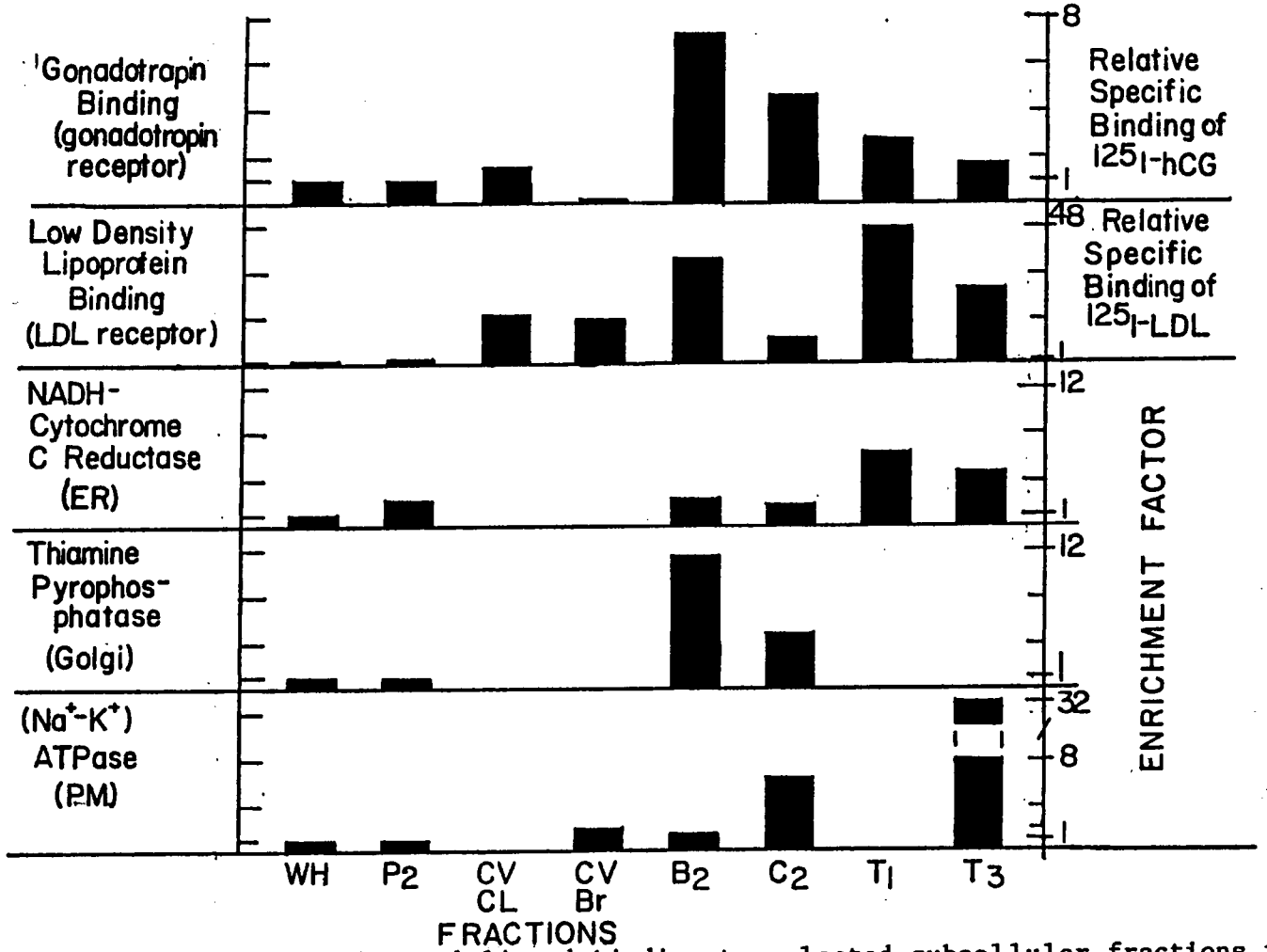

FIGURE 3. Marker enzyme activities and ligand binding to selected subcellular fractions from Table 2. SA, Specific activity (enzyme activity [nmole product/min.] divided by mg of protein OR binding of ^{125}I-ligand [cpm] divided by mg of protein). Relative specific binding (SA of fraction divided by SA of whole homogenate). Enrichment factor (SA of enzyme for fraction divided SA of whole homogenate).

vesicle fraction derived from the corpus luteum was very pure. Analysis with electron microscopy using a negative stain revealed that greater than 97% of the membrane profiles observed in this fraction could be identified as coated vesicles with a mean diameter between 75-85 nm, Figure 4. Approximately 75% of the coated vesicles appear to contain intact membranes (translucent core) and 25% are probably empty clathrin cages (no translucent core). Analysis of this fraction with marker enzyme assays demonstrated that there was no detectable activity for any marker enzyme measured (Table 2 and Figure 3).

In contrast, the coated vesicle fraction derived from brain tissue was not so pure, even though greater than 95% of membrane profiles in this fraction could be identified as coated vesicles. However, there may have been contamination with plasma membrane vesicles since this fraction elicited a substantial amount of activity for $(Na^{+}-k^{+})$ATPase (3.1 nanomoles of products per min. per mg of protein).

Binding of ^{125}I-hCG

The specific binding of ^{125}I-hCG in all fractions is depicted in Table 1, page 101. The specific binding was determined by subtracting the non-specific binding (100-fold excess of unlabelled hCG) per mg protein from the total binding per mg of protein. Nonspecific binding was always less than 5% of total counts added. All subcellular fractions obtained from differential and density gradient centrifugation elicited binding of gonadotropin, Table 1, page 101, but those fractions enriched the most with either plasma membrane,

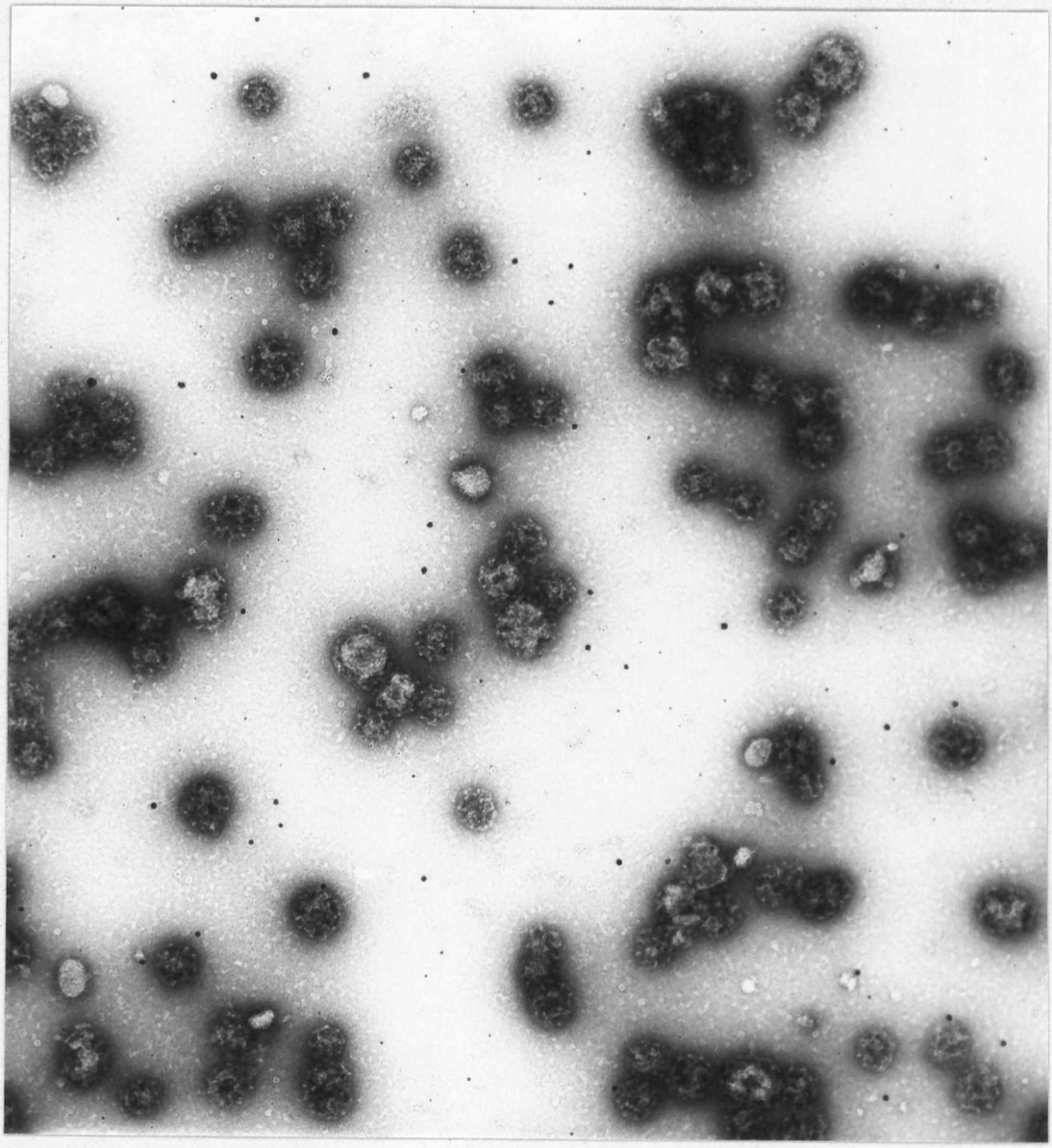

FIGURE 4. Electron micrograph of coated vesicle fraction. Magnification 70,000x.

Golgi membrane, and/or endoplasmic reticulum showed the greatest amount of binding of gonadotropin per mg of protein. The most pure of these membrane fractions were selected for comparison with the coated vesicles fractions in their ability to competitively bind gonadotropin, Table 2. Figure 3 illustrates the relative binding of these fractions in which the data is expressed as the enrichment factors (specific binding to fractions cpm/mg protein divided by specific binding of WH cpm/mg protein).

The fraction containing purified coated vesicles isolated from the corpus luteum elicited a gonadotropin binding of 78,733 cpm/mg of protein which is 1.51 times that of the whole homogenate (51,990 cpm/mg protein). In contrast, coated vesicles isolated from the brain (negative control) showed only a slight amount of gonadotropin binding 6,380 cpm/mg protein giving it an enrichment factor of 0.12 in comparison to the whole homogenate of the corpus luteum.

Coated vesicles isolated from corpora lutea at different stages of the reproductive cycle (Nonpregnancy, Early pregnancy, and Late pregnancy) also showed specific binding of gonadotropins, Table 3. Coated vesicles isolated from the corpus luteum from the nonpregnant stage (characterized as the absence of embryos in the uterus) elicited on the average a binding activity of 80,805 cpm/mg of protein (EF 1.75). Coated vesicles from the early pregnancy (intrauterine embryos 15 mm or less) and late pregnancy (intrauterine embryos greater than 20 cm) stages were depicted with binding activities of 94,455 cpm/mg protein (EF 9.7) and 139,358 cpm/mg protein (EF 6.85), respectively.

TABLE 3. Comparison of ligand binding to coated vesicles isolated from corpora lutea of different stages of the reproductive cycle. SA, specific activity (cpm/mg of protein); EF, enrichment factor (specific activity of CV fraction divided by specific activity of whole homogenate; WH, whole homogenate; CV, coated vesicles.

Stage of Cycle	Binding of ^{125}I-hCG		Binding of ^{125}I-LDL	
	SA x 10^3	EF	SA x 10^3	EF
Non Pregnancy				
WH	46.3	1	58.0	1
CV	80.8	1.74	3,904.0	67
Early Pregnancy				
WH	9.7	1	18.3	1
CV	94.4	9.7	2,783.3	152
Late Pregnancy				
WH	20.3	1	7.4	1
CV	139.4	6.85	4,928.8	664

For a positive control, gonadotropin binding to membrane fractions known to have gonadotropin receptors were examined. The B_2 fraction (Golgi & ER) which possessed the greatest amount of TPPase activity of any subcellular fraction also showed the greatest enrichment in gonadotropin binding (EF 7.4). The C2 fraction (PM-Golgi-ER), T_1 fraction (ER) and T_3 fraction (PM & ER) showed binding enrichment of 4.58, 2.75 and 1.75, respectively (Figure 3, page 105).

Isolation of LDL

Low density lipoproteins (density 1.019-1.026 gm/ml) were isolated according to the procedure described earlier. Analysis of the LDL fraction by negative stain electron microscopy revealed that it was a homogeneous fraction with particles of 10 nm in diameter, Figure 5. Agarose gel electrophoresis performed on the LDL fraction and stained for lipids demonstrated that this fraction consisted of a lipid moiety that migrated as one band that was different from HDL or other serum proteins, Figure 6. This purified LDL fraction was labeled with ^{125}I and used in binding studies.

Binding of ^{125}I-LDL

To measure LDL receptor activity in coated vesicle and other membrane fractions, fractions were incubated with ^{125}I-LDL in the presence and absence of nonlabelled LDL according to the procedures described in the "Material and Methods" section. The specific binding (cpm/mg of protein) for each fraction was determined by subtracting nonspecific binding (presence of cold LDL) from the total binding

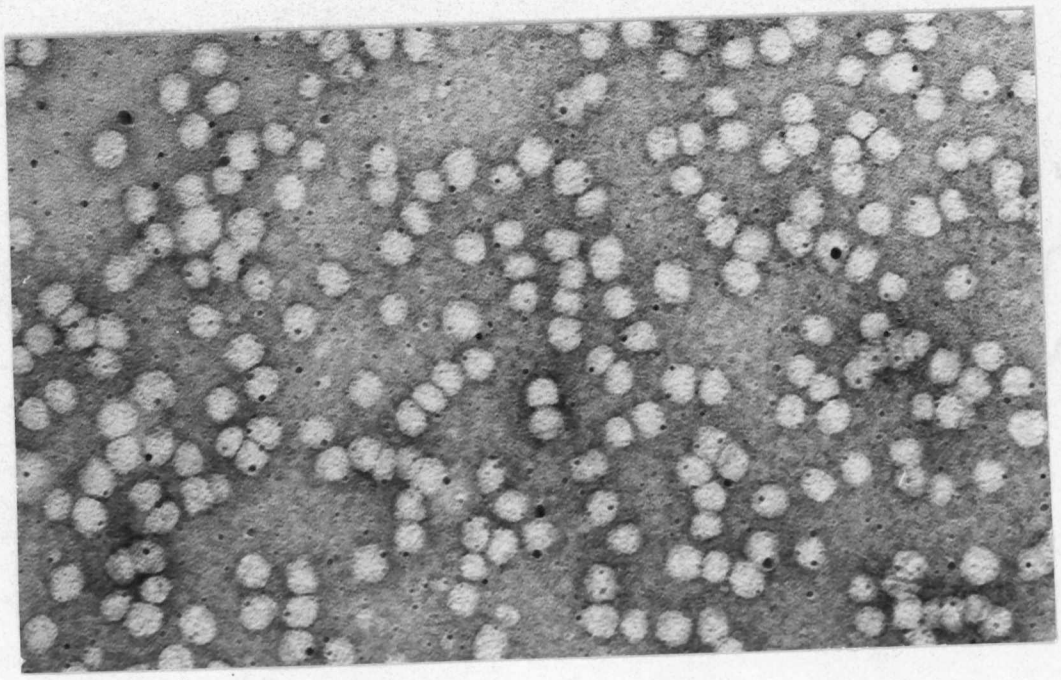

FIGURE 5. Electron micrograph of purified LDL. Magnification 198,000x.

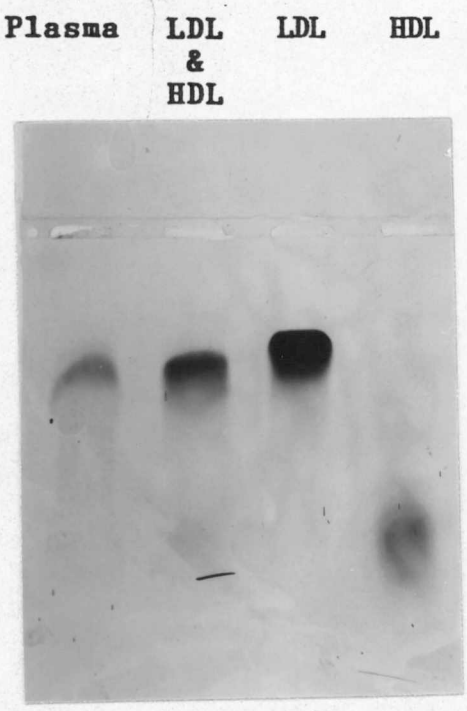

FIGURE 6. Agarose gel electrophoresis of LDL and HDL fractions. LDL, low density lipoprotein; HDL, high density lipoprotein.

(absence of cold LDL). Nonspecific binding was always less than 10% of the total counts. Table 1, page 101, shows binding activity to all fractions obtained during the isolation procedure for membrane organelles along with the coated vesicle fraction. Table 2, page 104 shows binding activity to selected fractions from Table 1, page 101 known to be enriched in either plasma membrane, Golgi membrane, endoplasmic reticulum, or a combination of membranes along with the coated vesicle fractions. Figure 3, page 105, depicts the enrichment factor of the LDL receptor for each of the selected fractions from Table 2, page 104 (enrichment factor (EF), specific binding of fraction divided by specific binding of whole homogenate (WH)).

According to the data the coated vesicle fraction purified from the corpus luteum possessed a binding activity of 825,862 cpm/mg of protein (EF 17.4). Coated vesicles isolated from the brain also possessed enriched binding activity, 747,495 cpm/mg of protein.

In a separate experiment using ^{125}I-LDL of greater specific activity, the binding activity of coated vesicles isolated from the corpus luteum from different stages of the reproductive cycle were examined, Table 3. Coated vesicles from the nonpregnancy stage possessed a binding activity of 3,903,922 cpm/mg protein (EF 67). In early pregnancy the binding activity attributed to coated vesicles was 2,783,220 cpm/mg protein (EF 152). Coated vesicles isolated from the corpus luteum from the late stage of pregnancy exhibit the greatest binding activity of any stage of the reproductive cycle 4,928,564 cpm/mg protein (EF 664). Taken together the data indicates that

coated vesicles from any stage of the reproductive cycle possess many unbound LDL receptors.

For a positive control, LDL binding to membrane fractions suspected in having LDL receptors were examined, Figure 3, page 105. Of all fractions examined, T_1 which possessed the greatest enrichment of ER, also possessed the greatest binding activity of LDL, 2,266,172 cpm/mg protein (EF 47.9). The fraction that was enriched the most with Golgi membranes B_2, (Golgi & ER), contained the second highest amount of binding activity, 1,755,800 cpm/mg protein (EF 37) and the T_3 fraction (PM & ER) also contained a relatively high amount of binding activity 1,273,363 cpm/mg of protein (EF 25.7).

Ligand Binding to Pretreated Coated Vesicles

To determine whether the receptors for hCG and LDL associated with coated vesicles are oriented to face the outside or inside, the coated vesicles were treated with detergent and/or proteases to unmask or destroy receptors prior to binding with either ^{125}I-LDL or ^{125}I-hCG.

Three treatment groups were employed to determine the orientation of receptors. Treatment group A: coated vesicles were incubated in the presence of detergent and proteases. In this group all receptors on either the inside or outside of the vesicles would be destroyed. This group served as a nonspecific control. Treatment group B: coated vesicles were incubated in the presence of proteases. Under this condition all receptors oriented to face the outer surface of the

coated vesicle would be destroyed. Those receptors oriented to face the inside of the vesicle would be protected from proteolytic digestion since proteases normally cannot penetrate membranes. To measure the binding activity of those receptors localized on the inside of the vesicle, binding assays were performed in the presence of detergent. Treatment group C: coated vesicles were not pretreated with proteases. All receptors associated with the coated vesicles whether localized on the inside or outside under this condition would remain intact and functional. Binding studies performed on coated vesicles from treatment group C were incubated in the presence of detergent to measure the total binding activity of receptors localized on the inside and outside or in the absence of detergent to measure binding activity of receptors localized on the outside of the vesicle. The summary of the procedure and results are depicted in Figure 7.

^{125}I-hCG Binding

The binding of gonadotropin to coated vesicles isolated from the corpus luteum is shown in panel A of Figure 7. The total binding activity of gonadotropin receptors localized on the inside and outside of the coated vesicle membrane was 95,894 cpm/mg protein (group C & Detergent). The binding activity of receptors presumed to be localized on the outside of the coated vesicles was 51,920 cpm/mg protein (group C & No Detergent). The binding activity of receptors localized on the inside was 43,466 cpm/mg protein (group B & Detergent). This data indicates that approximately 45% of the gonadotropin receptors appear to be oriented to face the inside of the

Binding ^{125}I-hCG to Coated Vesicles					Panel A Binding to CV from corpus luteum	Specific Binding	Panel B Binding to CV from Brain	Specific Binding
	Pretreatment	Conditions of Assay			cpm/mg	cpm/mg	cpm/mg	cpm/mg
Group A	Detergent and	50ul	+Detergent		21,920	18,906	18,371	16,000
100 1	Enzymes	50ul	+Detergent	+hCG	3,013		2,371	
Group B	Enzymes	50ul	+Detergent		46,053	43,466	8,037	5,496
200 1		50ul	+Detergent	+hCG	2,586		2,541	
		50ul			21,546	16,133	15,887	9,412
		50ul		+hCG	5,413		6,475	
Group C		50ul	+Detergent		119,440	95,894	19,482	1,374
200 1		50ul	+Detergent	+hCG	23,346		18,108	
		50ul			71,360	51,920	21,835	9,769
		50ul		+hCG	19,440		12,065	

Binding ^{125}I-LDL to Coated Vesicles					Panel C Binding to CV from corpus luteum	Specific Binding	Panel D Binding to CV from Brain	Specific Binding
	Pretreatment	Conditions of Assay			cpm/mg	cpm/mg	cpm/mg	cpm/mg
Group A	Detergent and	50ul	Detergent		2,800,000	2,347,000		
100 1	Enzymes	50ul	Detergent	+LDL	453,000			
Group B	Enzymes	50ul	Detergent		15,386,000	14,933,000		
200 1		50ul	Detergent	+LDL	453,000			
		50ul			853,000	480,000		
		50ul		+LDL	373,000			
Group C		50ul	Detergent		15,546,000	14,906,000	16,376,000	15,906,000
200 1		50ul	Detergent	+LDL	640,000		470,000	
		50ul			1,120,000	560,000	602,000	57,000
		50ul		+LDL	560,000		545,000	

FIGURE 7. Binding of ^{125}I-hCG and ^{125}I-LDL to pretreated coated vesicles isolated from either the corpus luteum or the brain. Details of treatments are given in "Material and Methods" section.

coated vesicle membrane. In contrast, coated vesicles isolated from the brain demonstrated no appreciable amount of gonadotropin binding, panel B, Figure 7. The data shows that all binding activity measured with coated vesicles isolated from the brain are at values less than the nonspecific binding attributed to coated vesicles isolated from the corpus luteum (panel A, group A).

^{125}I-LDL Binding

The binding of LDL to coated vesicles isolated from the corpus luteum is shown in panel C, Figure 7. The total binding of LDL (inside and outside) that can be attributed to coated vesicles is 14,906,000 cpm/mg protein (group C & Detergent). Group C (No Detergent) is binding that can be attributed to receptors localized on the outside 560,000 cpm/mg protein and group B (Detergent) depicts the binding activity of receptors localized on the inside of the coated vesicle membrane 14,933,000. Similarly, coated vesicles isolated from the brain also demonstrated binding activity of LDL (panel D, Figure 7) in which nearly all of the LDL receptors can also be localized on the inside of the coated vesicle. These data indicate that nearly all of LDL receptors, approximately 96%, were not available for binding with LDL unless the coated vesicles were pretreated with detergent. Presumably the LDL receptors are oriented to face the inside of the coated vesicle and/or the receptors are oriented to face the outside of the membrane, but are masked by a protein or a lipid that is removed by detergent treatment.

IV. DISCUSSION

The data obtained from the studies reported in this paper indicate that purified preparations of coated vesicles from the corpus luteum of the pig possess an abundant supply of receptors for both low density lipoprotein (LDL) and luteinizing hormone (LH). The scientific validity of this conclusion, however, is dependent on a number of factors, principle of which is the real purity of the coated vesicle fraction obtained by the centrifugation procedure. Analysis of this fraction by negative stain electron microscopy revealed that greater than 97% of all membrane profiles observed could be identified as coated vesicles. When this fraction was also surveyed for the activities of marker enzymes from possible membrane contaminants no detectable amounts of activity were measured. The results from both electron microscopy and marker enzyme assays correlate well with one another and provide evidence for reasonable homogeneity of the coated vesicle fraction. Thus any binding activity for either ^{125}I-LDL or ^{125}I-hCG detected in this fraction can probably be attributed to coated vesicles.

LDL receptors detected in the purified coated vesicle fraction represent a 17.4 fold enrichment over the whole homogenate on a mg protein basis. In comparison, the (Golgi & ER) fraction, ER fraction and (PM & ER) fraction isolated from the centrifugation process, possessed greater enrichments of LDL receptors than the coated vesicle fraction, enrichment factor of 37, 47 and 25, respectively. However, this comparison of binding activity on a mg protein basis may not

represent an accurate picture since over 60% of the protein composing the coated vesicle fraction is from the clathrin protein cage that surrounds each coated vesicle (45, 91, 121, 122). A more accurate estimate of the enrichment factor for the coated vesicle fraction based on coated vesicle membrane only is approximately 34 fold. This value is similar to what was estimated for the (Golgi & ER) fraction.

LH receptors present in the purified coated vesicle fraction elicited only a 1.5 fold enrichment over the whole homogenate. Whereas, the (Golgi & ER) fraction showed nearly five times as great an enrichment (EF 7.44). Although the amount of LH receptors in the coated vesicle fraction was small when compared to the other membrane fractions, we think this was an accurate representation and not an artifact for several reasons. First, the coated vesicle fraction was very homogeneous and there was no evidence of contamination from any other membrane organelle. All ^{125}I-hCG binding to this fraction could thus be ascribed to the coated vesicles. Second, as a negative control, coated vesicles isolated from the brain were examined for their ability to competitively bind ^{125}I-hCG. No LH receptors were detected. If the gonadotropin binding to coated vesicles from the corpus luteum was an artifact, then a proportionate amount of binding to coated vesicles isolated from the brain would also have been detected. Third, the binding activity of ^{125}I-hCG was examined in coated vesicles isolated from the corpus luteum at three different stages of the reproductive cycle, nonpregnancy, early pregnancy and late pregnancy. In all stages, LH receptors were localized in the

coated vesicles but the proportionate amount varied. Coated vesicles from the early pregnancy stage exhibited nearly a 10 fold enrichment of LH receptors whereas those from the nonpregnancy showed only a 1.75 fold enrichment. If the ^{125}I-hCG binding to coated vesicles from the corpus luteum was an artifact, a consistent and proportionate amount of binding activity would probably be measured in coated vesicles from the corpus luteum of any stage of the reproductive cycle. The variability in the amount of gonadotropin binding to coated vesicles from the corpus luteum at different stages of the reproductive cycle probably represents differences in functional states of the corpus luteum. Therefore, the low binding of gonadotropin to coated vesicles from the corpus luteum from the nonpregnant stage of the reproductive cycle is most likely a natural condition.

Further analysis of the binding of LDL and hCG to coated vesicles revealed that 97% of the LDL receptors and only 45% of the LH receptors appear to be localized on the inside of the coated vesicle and are only available to binding with ligand in the presence of detergent. The low percentage of hCG binding activity associated with the inside of the vesicles was a surprise. We expected a percentage value similar to that for LDL binding. One possible explanation for this is that the membrane of the coated vesicle was partially damaged during the isolation procedure or is naturally porous. If holes or pores present in the coated vesicle membrane were large enough to allow for penetration of ^{125}I-hCG into the vesicle, then detergents would not be required to permealize the coated vesicle membrane and

the LH receptors present on the inside of the vesicle would be available for binding. Under this condition no distinction could be made between LH receptors oriented to the inside of the coated vesicle or to the outside. This seems like a reasonable explanation but several questions need to be addressed. First, in other experiments coated vesicles were pretreated with proteases prior to binding with hCG to destroy any outer surface oriented receptors. Under this circumstance all the receptors that were oriented to the inside of the membrane should have been protected from the proteolysis and should have only been available to binding with hCG when detergents were added. If, on the other hand, the membrane of the coated vesicles was damaged and had holes or was naturally porous, then all LH receptors whether on the inside or outside of the coated vesicles would most likely have been destroyed by the proteases and consequently there should have been no binding of gonadotropins. However, if the speculative holes or pores in the coated vesicle membrane were small and selective, then perhaps only a small amount of proteases or hCG would actually leak into the coated vesicle in the absence of detergents. Under this condition, an incomplete digestion of gonadotropin receptors would probably occur or a small amount of hCG would bind to its receptor in the absence of detergent. Second, nearly 97% of all LDL receptors in coated vesicles appear to be oriented towards the inside of the vesicle and were only available to binding with ^{125}I-LDL when the membranes were permealized with detergents. If the membrane of the coated vesicles was damaged and

had holes or was naturally porous, then no detergents should have been required for binding of LDL. However, if the speculative holes or pores in membranes of coated vesicles were small, perhaps only a small protein like the hCG (MW 34,000) could penetrate but not a larger protein-lipid aggregate like LDL (MW greater than 3,000,000). Although the presence of holes or pores in the membrane of coated vesicles seems like a plausible explanation for the unexpected results of gonadotropin receptor distribution, further studies, such as longer incubation times of protease treatments or hCG binding assays in the absence of detergents, are needed to confirm this possibility.

An alternate explanation for the low percentage of gonadotropin binding activity and the high percentage LDL binding activity associated with the inside of coated vesicles is that there may be natural differences in the structure and way in which these two receptors are oriented in the membrane. The LDL receptor is a large transmembrane protein with a MW of 160,000 (153,162). Goldstein et al. (29, 66, 148, 178) have mapped out several structural and functional domains on this receptor. A hydrophobic domain anchors the receptor in the membrane. On one end of the receptor is another domain for binding LDL and on the opposite end of the receptor on the other side of the membrane in which it is anchored is the cytoplasmic domain which plays an important role in clustering in coated pits, either through interaction with clathrin itself or with some protein that is associated with clathrin on the cytoplasmic side of the membrane. Studies on intact cells indicate that the LDL receptor

localized in coated pits is oriented in the membrane so that the LDL binding domain faces to the outside of the cell or inside of the coated pit and the cytoplasmic domain faces the cytoplasmic side of the coated pit and most likely is linked to the clathrin cage or some protein associated with the cage (28, 65). The linkage of the LDL receptors to the clathrin cage, if it does occur, would probably maintain the way in which these receptors are oriented in the membrane by constraining the receptor from any lateral or vectoral migration. After internalization of coated pits, LDL receptors within newly formed coated vesicles would probably be oriented in the membrane in this same way. However, we cannot yet rule out the possibility that in coated vesicles the LDL receptors reposition themselves in the membrane and become oriented towards the outside, but are masked by clathrin or some other protein that is removed when treated with detergents.

The LH receptor has an estimated molecular weight of 120,000-200,000 (50, 142, 174, 175). The structure of and the way in which this receptor is oriented in the membrane is even less clear (142). Studies on photoaffinity-labeled or affinity-cross-linked receptors indicate that the receptor may be oligomeric with at least four different subunits (84, 140, 142). Recent studies performed by Wimalasena suggest that purified porcine LH receptor is an oligomer of identical subunits of 86,000 MW (176). The way in which these subunits of the receptor are arranged in the plasma membrane is unknown. Presumably, however, a portion of the receptor would face

toward the outside of the cell and bind LH or hCG and a hydrophobic portion would anchor the receptor in the membrane. Whether there is also a cytoplasmic domain on the LH receptor that can play a role in trapping of the receptors into coated pits, as was the case with the LDL receptors, is not known. But if there is, then it may not be functional or least very effective until the receptor is bound with LH since only at this time are the LH receptors observed to move over the cell surface to coated pits where they are trapped and internalized. The arrangement of the LH receptor in the membrane of coated vesicles from endocytosis or in coated vesicles derived from the Golgi apparatus carrying newly synthesized receptors is unknown. If the coated vesicle membrane is intact with no holes or pores, contrary to what was earlier suggested, then the data suggests that approximately half of the gonadotropin receptors in the coated vesicle membrane are oriented to the outside of the vesicle. If all the gonadotropin receptors that are present/or will be present on the outer plasma membrane are oriented to the outside of the cell, as is generally assumed, then this suggests that a rearrangement of the gonadotropin receptors in the coated vesicle membrane must occur prior to/or during insertion into the plasma membrane. The major criticism with this is that it involves a vectoral rearrangement (flip-flop) of the receptor. This generally is assumed to be thermodynamically non-feasible and therefore, some other mechanism must be operating that explains the equal distribution of gonadotropin receptors across the coated vesicle membrane. The first explanation, discussed earlier, seems to be the

more reasonable.

Although we were the first to show that isolated coated vesicles from the corpus luteum possess receptors for both LH and LDL, the presence of ligand receptors in coated vesicles is not at all a new finding. Receptors for a variety of ligands have been localized in isolated coated vesicles from a variety of tissue types. For example, in the adrenal cortex which is a steroid secreting tissue like the corpus luteum, isolated coated vesicles were found to possess LDL receptors (108). In brain tissue receptors have been identified in coated vesicles for mannose 6 phosphate (31, 33) and from our study, LDL. Coated vesicles isolated from other tissues also show abundant receptors: liver, mannose 6 phosphate (31, 33) and asialoglycoprotein (62, 63); muscle, acetyl-choline (20); placenta, transferrin (22, 125) and immunoglobullins (125). Receptors in coated vesicles are probably a universal phenomenon and most likely as coated vesicles are isolated from other cell types, receptors characteristic of the cell type will be found in them.

One apparent role of coated vesicles is to transport membrane and membrane proteins, such as receptors, from one subcellular compartment to another (10, 59, 69, 80, 116, 124, 143, 144, 146, 147). This presupposition is based in part on the mechanism of formation and fate of coated vesicles as deduced from electron microscopy. In the formation of coated vesicles, clathrin, the major protein consitituent of the coat structure (45, 91, 121, 122) combines with two other clathrin molecules along with three 100,000 MW proteins to form a

three armed structure called a triskelion (94, 163, 164). Triskelions are thought to assemble together to form larger pentagon and hexagon structural units on the membrane (45, 46, 57, 65, 81, 90, 122, 124, 133, 177). As more clathrin pentagons and hexagons are added to the growing superstructure, structural constraints force the underlying membrane to invaginate and eventually pinch off to form coated vesicles. Coated vesicles appear to be transported to target membrane compartments where their clathrin coat is lost prior to membrane fusion and subsequent delivery of membrane constituents (3, 10, 23, 52, 59, 65, 98, 120, 124, 152). Any membrane protein associated with the coated pit/vesicle would also be sequestered along with the membrane during the formation of coated vesicle. Thus in this way, coated vesicles can serve to transport membrane receptors from one subcellular compartment to another subcellular compartment.

In granulosa lutein cells, our current hypothesis for the mechanism in which LDL and LH receptors are renewed on the cell surface is that there is an intracellular source of both LDL and LH receptors which are transported and inserted into the outer cell surface via coated vesicles. The data presented in this paper support this hypothesis in several ways. First, Golgi membrane and endoplasmic reticulum enriched subcellular fractions derived from the granulosa lutein cell possess receptors for both LDL and LH. In particular, the subcellular fraction that was enriched with the greatest amount of Golgi membrane also exhibited the greatest amount of binding activity of ^{125}I-hCG compared to any other subcellular

fraction. Presumably, the receptors within these organelles represent an intracellular pool of either newly synthesized or recycled receptors that could be the source of receptors that are used to replace those that are lost from the cell surface. Second, in granulosa lutein cells, secretory granules and coated vesicles are the only membrane bound structures observed to be derived from the Golgi apparatus, the presumptive intracellular source of receptors, that later are transported and inserted into the plasma membrane. Earlier studies from our laboratory demonstrated that the membrane that surrounds the secretory granule does not possess gonadotropin receptors and therefore could not be the vehicle for receptor transport. The coated vesicles, on the other hand, have been shown conclusively in this paper to possess both LDL and LH receptors and therefore have been implicated as the vehicle for LDL and LH receptor transport.

Although coated vesicles isolated from granulosa lutein cells possess LDL and LH receptors, it is not known, however, whether the coated vesicles are actually responsible for inserting new receptors into the plasma membrane. Coated vesicles also have been demonstrated to be involved with the internalization of both LDL and LH receptors during endocytosis in granulosa lutein cells (1, 4, 38, 39). Current methods do not allow us to distinguish between these two functional types of coated vesicles, endocytotic vs. exocytotic. However, Friend and Faraquhar (59) have reported that in epithelial cells there are two classes of coated vesicles based on size. Coated vesicles derived

from the Golgi apparatus have a mean diameter of 75nm whereas those that are derived fron invagination of coated pits during endocytosis are approximately 100nm in diameter. Based on this one criteria of size, coated vesicles isolated from the corpus luteum in this study which have an average diameter of 75-85 nm were probably derived from the Golgi body. If this is true, then coated vesicles may be involved in transporting the LDL and LH receptors from the Golgi area in the cell to the outer cell surface where they are inserted.

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PART 4

SUMMARY AND CONCLUSION

The large granulosa lutein cells of the corpus luteum of the ovary in the pig synthesize and secrete progesterone (1, 15, 38, 59, 60, 79, 111, 113, 116). Receptors that bind luteinizing hormone (LH) (1, 5-8, 11, 13, 21, 22, 28, 29, 30, 37, 41, 52, 55, 66, 68, 77, 78, 97, 100, 101, 103) and others that bind low density lipoproteins (LDL) (9, 14, 15, 24, 31, 54, 63, 75, 85, 88, 102, 115, 122) play important roles in the regulation of the secretory event. Both types of receptors, when bound with their specific ligand, are internalized by endocytosis (1, 3, 5, 30, 31, 88). Continuous internalization and destruction would eventually result in loss of the cell's ability to respond to ligand. This is not, however, what is observed. This cell type maintains its ability to respond to new challanges of ligand throughout its existence. (38, 57, 64, 83, 119). Therefore, some mechanism must operate that replaces those receptors lost from the cell surface.

Earlier studies by the author (Gonadotropin Receptors in the Ovarian Granulosa Lutein Cell of the Pregnant Pig, Master's Thesis, June 1982) revealed that a pool of unbound gonadotropin receptors was located within the cell and that subcellular fractions enriched with Golgi body membranes or endoplasmic reticulum possessed gonadotropin receptors, expecially the Golgi body fractions. A presupposition was made that the intracellular receptors located in these organelles were the source of new receptors but the mechanism of transport of these receptors from within the cell to the outer surface was unknown.

The purpose of the research presented in this dissertation was to

begin to determine if gonadotropin receptors localized within the Golgi apparatus are translocated and, if so, how they are inserted into the plasma membrane of this cell type.

Examination of this cell type by electron microscopy revealed that two different types of membrane structures, secretory granules and coated vesicles, appear to orginate from the Golgi apparatus and later insert into the plasma membrane (1, 17, 32, 34, 46, 47, 80, 86, 87, 112, 113). Either type could then potentially function as a vehicle for transporting receptors to the plasmalemma. The membrane surrounding the secretory granule was originally hypothesized as the most reasonable candidate for several reasons. First, during the luteal phase of the estrous cycle, the principal membrane-bound structure observed to be derived from the Golgi apparatus in this cell type was the secretory granule. Whereas, very few coated vesicles can be found at or near the vicinity of the Golgi apparatus during this time. Second, membranes of secretory granules from several other cell types were shown to possess hormone receptors. Somatostatin receptors have been localized in secretory granules isolated from the pituitary gonadotrope (33, 117), as well as the pancreatic islet cells (81, 117, 118). The secretory granules from the gonadotropes also possess GNRH receptors (40). However, after extensive analysis, gonadotropin receptors were not observed in the membrane surrounding secretory granules in granulosa lutein cells and, therefore, are probably not involved in receptor translocation.

A second type of membrane structure, coated vesicles, is also

present in the regions of the Golgi. Radioisotopic labeling studies had shown that they too probably moved to the cell surface in prodigious numbers and become incorporated into the plasma membrane, particularly during the earliest period of the luteal phase of the cycle (1). Although coated vesicles are less prominent in these cells during later times of the luteal phase, their presence portends the possibility of their involvement in receptor translocation

The study presented in this dissertation was designed to determine whether coated vesicles from the corpus luteum could participate in receptor translocation. Coated vesicles isolated from this tissue by a combination of differential sedimentation and density gradient centrifugation techniques were relatively pure as judged by electron microscopy and by the lack of marker enzyme activities from potential contaminating organelles. Radioreceptor assays performed with ^{125}I-LDL and ^{125}I-hCG demonstrated high affinity binding sites for these two ligands within coated vesicles. Consequently, the conclusion was made that coated vesicles probably function as a vehicle for LH and LDL receptor translocation in granulosa lutein cells.

Controversy exists whether coated vesicles are actually involved in the translocation of membrane proteins from the Golgi apparatus to the plasma membrane. In cultured fibroblasts infected with vesicular stomatitis virus (VSV), Wehaland (1982) (123) demonstrated using immunoflourescence and high resolution electron microscopy that the newly synthesized VSV G protein was localized in the endoplasmic

reticulum, Golgi body and plasma membrane, but not in coated vesicles at or near the Golgi apparatus of the cell. Futhermore, when antibody to clathrin, the major protein constituent of coated vesicles, was injected into these cells, no interference in the insertion of newly synthesized VSV G protein into the plasma membrane was observed. Presumably the antibody would interfer with coated vesicle formation and thereby block the transport of G protein to the PM if coated vesicles were involved. Other groups at this time however, demonstrated that coated vesicles possessed manose 6-phosphate receptors and were responsible for trapping and translocating newly synthesized lysosomal enzymes from the Golgi body to the lysosome (25, 50, 110, 126). Several investigators believed coated vesicles derived from the Golgi were identical in structure and function, which would make it difficult to imagine how the same coated vesicle could participate in both the translocation of lysosomal enzymes to the lysosomes and the translocation of the VSV-G protein from the endoplasmic reticulum to the Golgi and then to the plasma membrane. Consequently, Wehland concluded that coated vesicles were not involved in the translocation of newly synthesized viral G protein in infected fibroblasts.

More recent evidence, however, indicates that the Golgi apparatus is involved in the processing, sorting, and packaging of lysosomal enzymes as well as secretory protein and membrane proteins (42, 43, 109). The coated vesicles derived from the Golgi appear to play a central role in this sorting event and in the delivery of these

proteins to their proper target organelles. The mechanism in which this is accomplished has by no means been worked out, but several possibilities seem reasonable.

One mechanism that could operate is that the Golgi contains several classes of coated buds. Each class is responsible for recognizing and sequestering a specific group of proteins such as lysosomal, secretory, or membrane proteins. Within the membrane of these coated buds, adapter molecules, as suggested by Pierce and Bretsher (93), may be present to target newly formed coated vesicles to their proper destination. In support of this model, Kedersha (71) demonstrated that coated vesicles derived from the liver can be separated into at least five distinct subpopulations using agarose electrophoresis. Coated vesicles from all subpopulations have identical coat proteins as determined by SDS PAGE. However, several non-coat proteins (manose 6-phosphate receptor, asialoglycoprotein receptor and other proteins) appear to segregate exclusively with different subpopulations of coated vesicles as resolved by two dimensional electrophoresis. In a different study, Pfeffer and Kelly (98) have identified two possible adapter molecules (MW 38,000 and 29,000) in subpopulations of brain coated vesicles isolated by antibody specific to transmembrane synaptic glycoprotein. Using protein solubilization techniques, Weidenmann (124, 125) has extended the number of candidate adapter molecules in brain coated vesicles to four (MW 38,000, 29,000, 24,000, 10,000). Helmy (58), using a density shift technique, was able to separate endocytotic vs. exocytotic

liver coated vesicles from each other. No difference in protein content was found between these two subpopulations. However, a difference in the ratio of cholesterol to phospholipid was evident. Although no significant difference in solubilized protein content was found between these two subpopulations, there is the possibility that the speculated protein adapter molecule(s) is present in relatively few copies, and therefore escaped detection by Helmy's techniques. Another possibility is that the adapter molecule, if it really does exist, is not a protein but rather a lipid. The adapter molecules could also represent subtle conformational changes between otherwise identical protein molecules caused by phosphorylation or acetylation. After considering all data, there appears to be distinct subpopulations of coated vesicles within a cell, and some of these subpopulations of coated vesicles, at least those from the brain, have unique non-coat proteins that may determine the contents of and direct individual vesicles to target organelles.

A second possible mechanism in which the Golgi could sort lysosomal enzymes, secretory and membrane proteins from each other and then deliver these products to their proper destiny might involve temporal regulation of the secretion of these different proteins. In this mechanism, the cell would synthesize either lysosomal enzymes, secretory or membrane proteins but not all three at any one time. The newly synthesized proteins would then be sequestered by coated buds in the Golgi apparatus. Newly formed coated vesicles would then transport the product to its target organelles. With time the cell

would stop synthesizing whatever protein it happened to be producing and shift its synthetic machinery to making new types of proteins. Eventually the cell will synthesize the original protein again and the whole cycle will repeat. In this way, each wave of newly synthesized proteins that passes through the Golgi and is sequestered by coated vesicles can very effectively be separated or sorted from each other. An example of temporal regulation of protein synthesis and secretion is found with the PMN leukocyte (16). In this cell type, the Golgi complex is responsible for packaging two types of secretory granules which are very different in their contents (one lysosomal, one not). The obvious problem of sorting proteins is resolved by making two sets of granules in two separate waves of protein synthesis. One type of granules is made and completed before the other has started. In this way, the contents of the two granules are separated from each other by time. Interestingly, opposite sides of the Golgi apparatus are used in the packaging of these two different types of granules.

If a mechanism of temporal regulation of secretory proteins were to operate in other cells, it certainly would simplify the difficult problem of sorting secretory proteins that is usually attributed to the Golgi complex. This mechanism, however, does not resolve the issue on how coated vesicles derived from the Golgi which supposedly would have different protein cargoes are translocated to their proper destination. The simplest resolution, I think, would be that some type of adapter molecule present in the coated vesicle membrane directs these vesicles to their proper targets. So, it seems that the

former mechanism described earlier for coated vesicle targeting is the more reasonable, the better supported and more popular.

In conclusion, I am proposing separate models for the renewal of LDL and LH receptors in granulosa lutein cells. These models are illustrated in Figure 1, LDL receptor renewal, and Figure 2, LH receptor renewal. In both models coated vesicles play a central role in the translocation of receptors from one membrane compartment to another during either exocytosis or endocytosis.

Figure 1 illustrates the proposed mechanism that may operate to renew LDL receptors. This hypothetical model, with a few exceptions, is based almost entirely on the model of LDL receptor renewal first proposed by Goldstein and Brown for fibroblasts (51a, 24a). Referring to Figure 1, LDL receptors are initially synthesized and glycosylated in the rough endoplasmic reticulum (RER), transported to the Golgi apparatus via coated vesicles where further modifications of sugars on the receptors occurs. Coated vesicles bud off from the Golgi, lose their coat, and then transport the newly synthesized receptors to the cell surface where they are inserted into the plasma membrane (PM). Unlike the fibroblast, the LDL receptors on granulosa lutein cells appear to be spread out over the cell surface in a diffuse pattern (88). After the receptors bind LDL, the LDL receptor complexes aggregate over coated pits and are internalized by endocytosis. Coated vesicles that form from this process transport the LDL-receptor complex to the endosome or prelysosomal compartment. Here an acidic pH triggers the dissociation of LDL from its receptor. The acronym

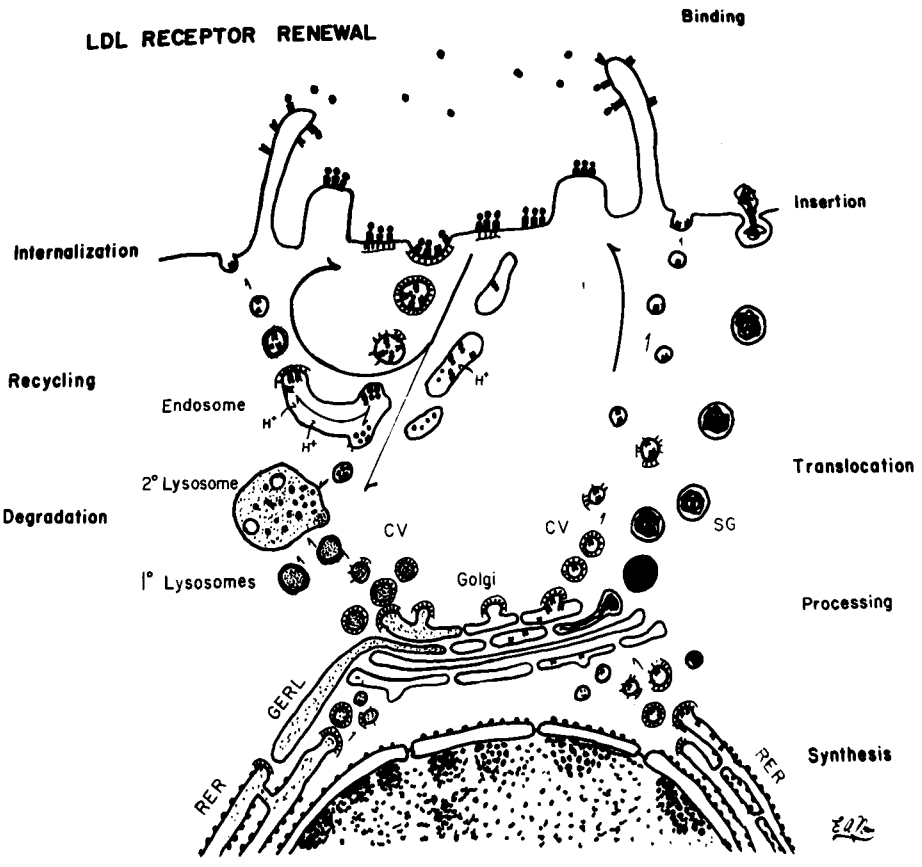

FIGURE 1. Hypothetical model of LDL receptor renewal of granulosa lutein cells.

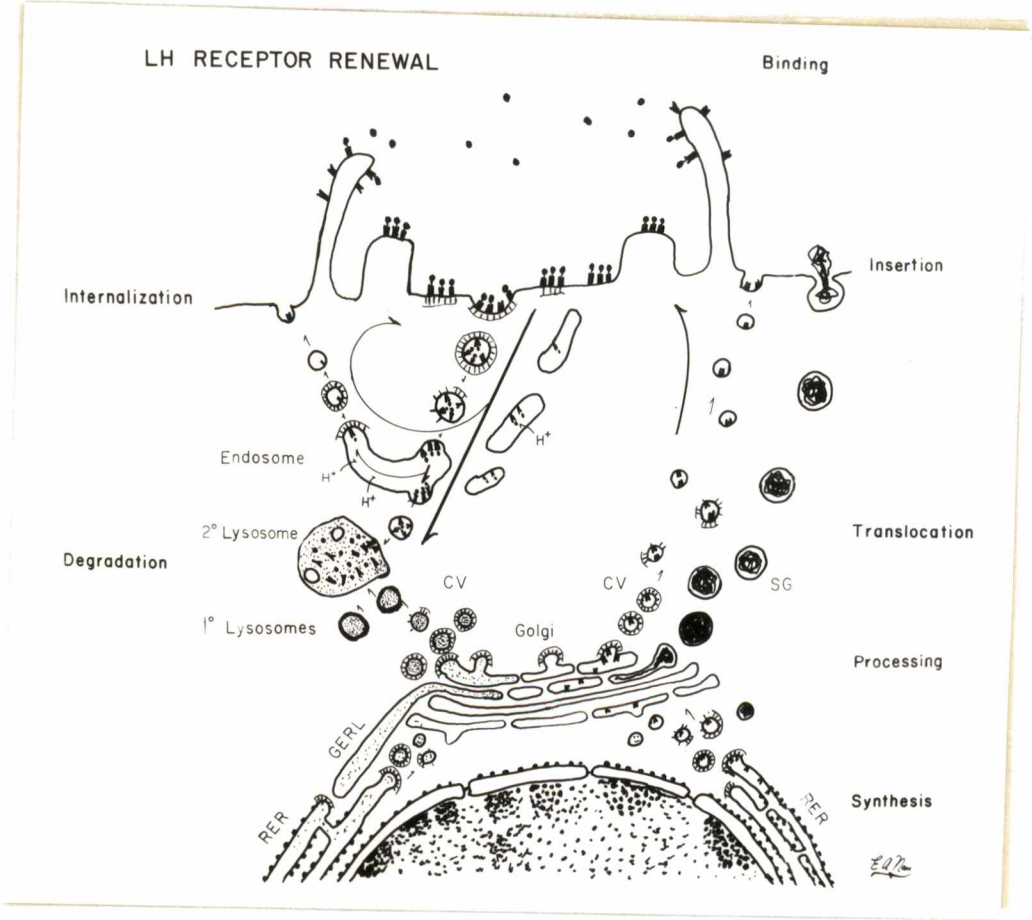

FIGURE 2. Hypothetical model of hCG receptor renewal in granulosa lutein cells.

CURL is used to denote the compartment of uncoupling of receptor and ligand. From this point on, LDL and its receptor usually follow different paths. The LDL is channeled to the lysosome for enzymatic degradation to release stored cholesterol. The uncoupled receptor, in contrast, is usually recycled to the cell surface for reutilization. However, eventually even the receptor is channeled to the lysosome for destruction. The number of LDL receptors on the cell surface is thought to reflect a balance between the destruction of internalized receptors, the recycling of receptors to the surface and the synthesis and insertion of new receptors into the PM. The recycling of receptors enables this cell type to reutilize and maintain larger numbers of receptors on its cell surface during times of rapid LDL uptake.

In contrast, LH receptor renewal appears to follow a slightly different mechanism as shown in Figure 2. Initially as with LDL receptors, the LH receptors are synthesized and glycosylated at the level of the RER, transported to the Golgi for further processing and eventually inserted in the PM. All through this course of events, coated vesicles are responsible for the translocation of the LH receptor from one membrane compartment to another. Once on the outer surface, the newly synthesized receptors spread out in a diffuse pattern but they are primarily concentrated over the surface of the microvilli. If the receptors become bound with LH or hCG, the LH-receptor complexes aggregate into small patches which slowly are moved to coated pits where they are internalized by endocytosis. I suspect

that only small quantities of unbound LH receptors are actually internalized unless they are first bound with hormone. The number of receptors internalized is usually proportional to the number of receptors bound with LH. In contrast, LDL receptors are internalized on a continual basis, at least in fibroblast, even though they may not be bound with LDL. After internalization, the coated vesicles that form lose their coat and fuse with the endosome compartment. Unlike the LDL-receptor complex, the LH-receptor complex probably does not dissociate because the pH in the endosome compartment (pH 6.0) is not acidic enough to cause effective uncoupling of LH from its receptor which usually requires a pH of less than 3.0. Ascoli (12) has provided evidence that in the rat the entire LH-receptor complex is channeled to the lysosome for destruction. There is very little evidence that internalized LH receptors are ever recycled to the cell surface.

The number of LH receptors on the cell surface of granulosa lutein cells consequently appears to be regulated by two opposing processes. The first is by the amount of LH available for binding to these cells. If granulosa cells are exposed to large doses of gonadotropins almost all the bound receptors will be lost from the cell surface by endocytosis. The second regulating process is the rate of synthesis and insertion of new receptors into the PM. Altering either process will greatly effect the number of LH receptors on the cell surface and consequently the ability of the cell to respond to new challenges of LH. This type of mechanism of receptor

renewal probably enables the cell to adjust quickly the number of LH receptors on its cell surface to a level appropriate to the physiological need.

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VITA

Eugene Alfred Nau was born in Omaha, Nebraska, on September 5, 1953. He attended elementary schools in that city but at the age of 10 his family moved to Littleton, Colorado, where he resumed his studies and eventually graduated from Littleton High School in June 1971. The following September he entered Arapahoe Community College as a part-time student. Working while attending ACC, Eugene was able to save enough money in a two year period to allow him to enroll at Colorado State University.

At CSU in the middle of his sophomore year Eugene had the fortune to meet Dr. John H. Abel, a professor in the Department of Physiology and Biophysics. Mesmerized by Dr. Abel's brilliance and enthusiasm for science, he began working in Dr. Abel's laboratory as an undergraduate research participant. It was in this environment with the constant exposure to Dr. Abel and the stimulating experience with research that Eugene decided to direct his life towards becoming a research scientist. In June 1977, Eugene received a Bachelor of Science degree in the field of Zoology.

In the following Fall, Eugene accepted a graduate research assistantship at CSU and began study towards a Master's degree under Dr. Abel's supervision. However, before he could complete his degree, Dr. Abel left CSU and accepted the position as head of the Department of Zoology at The University of Tennessee, Knoxville. Encouraged by Dr. Abel, Eugene withdrew from CSU, accepted a graduate teaching assistantship at The University of Tennessee and began study towards a

new Master of Science degree. While attending UT, Eugene received a Chancellor's citation for teaching excellence (Spring 1981) and in June 1982 he was awarded the Master of Science degree. Over the next five years, he completed his doctoral research, concentrating on the discipline of Cell Biology with a specialty in Cellular Endocrinology and graduated in June 1987.

Eugene Nau is currently working as a Post Doctoral Fellow at Lehigh University, Bethlehem, Pennsylvania. Eventually when he has completed his training, Eugene plans to apply for an academic position at a major institute known for its excellence in teaching and research.