To the Graduate Council:

I am submitting herewith a thesis written by Paul Dudley Gibson entitled "Interaction between Luteinizing Hormone and Prolactin Receptors in the Rat Ovary." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Thomas T. Chen, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

The Graduate School

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ABSTRACT

The loss of luteotropic receptors for prolactin (PRL) and luteinizing (LH) hormone in the rat corpus luteum under desensitizing conditions suggests a mechanism designed to reduce target cell responsiveness to excessive hormone stimulation. The mechanism involved in the coordinated loss of both receptors during down regulation is unknown but may involve cointernalization of LH and PRL receptors within the same endocytic vesicle. This may imply that a close physical association exists between luteotropic receptors in the rat corpus luteum. The present studies examine the kinetics and pattern of PRL and LH receptor loss and recovery in the corpus luteum of PMSG/hCG primed immature rats. Seven days after PMSG treatment rats were injected with either hCG, GnRH or $PGF_{2\alpha}$ and sacrificed at selected time points over a period of 8 days. A desensitizing dose (6-9µg) of hCG caused a 70-80% decrease in LH receptors within 24 hours. LH receptor levels remained low until day 2 but recovered to (or above 50%) control values by day 4. GnRH (2µg des Gly^{10},[D-Ala^{6}]GnRH ethylamide) caused LH receptors to decrease 40-50% by day 1 from which final receptor numbers slowly increased to peak levels at days 4 and 6. $PGF_{2\alpha}$ (1 and 2mg Lutalyse) caused LH receptors to decrease more slowly compared to hCG treatment. However, LH receptors continued to decrease and remained well below control values for the entire 8 day period. By contrast, the loss of lactogen receptors occurred as early as 6 hours after treatment with hCG, GnRH

or $PGF_{2\alpha}$. This was followed by a faster recovery when compared to LH receptors. These results indicate a similarity in loss and recovery of PRL and LH receptors in the rat corpus luteum. The association between LH and PRL receptors was further investigated by adding fluorescein-hCG and rhodamine-oPRL to cultures of rat granulosa-lutein cells and incubating at either 15 minutes or 2 hours at 37°C. Fluorescence from both fluorescein and rhodamine was localized as distinct patches in close proximity to each other on the surface and intracellularly. These results suggest that receptors for LH and PRL are functionally coupled and that during periods of continued hormonal stimulation, both receptors are internalized within the same endocytic vesicle.

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CHAPTER I

LITERATURE REVIEW

1. Role of Prolactin (PRL) in Reproductive Processes

Along with luteinizing hormone (LH), Prolactin (PRL) is regarded as a major luteotropic hormone in the rat and is required for a wide variety of functions during various physiological states.

In the pseudopregnant rat both diurnal and nocturnal surges of PRL have been observed (Smith et al., 1976) with peak nocturnal levels occurring 7 hours after the onset of the dark period. In rats treated with pregnant mare serum gonadotropin (PMSG) and human Chorionic Gonadotropin (hCG), progesterone production increased sharply from day 3 of pseudopregnancy. Day et al. (1980) found that the morning surge of PRL on day 3 of pseudopregnancy is essential for luteal survival and progesterone production.

In normal cycling rats high levels of progesterone are secreted at the beginning of diestrus. Functional regression of the corpus luteum occurs by day 4 or 5. The ultra short-lived life span of the rat corpus luteum can, however, be extended if rats are mated or subject to cervical stimulation which induces the pituitary gland to release two daily surges of PRL for approximately 12 days.

PRL is required not only for the prolongation of diestrus in both intact and hypophysectomized rats but also for the formation of deciduomata and the maintenance of pregnancy. In addition, PRL is

involved in growth and synthetic activity of the mammary gland and is implicated in the regulation of uterine function in the mink (Rose et al., 1983).

Hafiez et al. (1972) and Bartke et al. (1977) demonstrated PRL potentiation of a number of LH-stimulated responses including testicular growth, testosterone production and spermatogenesis. Similarly, PRL is involved in the increase in progesterone production by the corpus luteum (Döhler and Wuttke, 1970; Richards and Williams, 1976), a function acutely important in the prolongation of diestrus and maintenance of pregnancy. Armstrong and King (1971) suggested a possible role for PRL in inhibiting the conversion of progesterone to its metabolites so that high levels of the steroid in circulation ensure continued maintenance of specific physiological states.

However, in vitro studies by Armstrong et al. (1969) demonstrated that LH, and not PRL, stimulates acute release of progesterone, lending support to the hypothesis that PRL acts to maintain progesterone production in luteal cells that are already programmed for this activity. McNatty et al. (1974) implicated a possible role of PRL in maintaining cholesterol pools for the synthesis of progesterone in the ovary.

The action of PRL may be dependent on its level in circulation and on the number of specific PRL binding sites in target tissue. Although widely regarded as a major luteotropic hormone in the cycling rat, evidence of a luteolytic effect was reported (Wuttke and Meites, 1971; McDonald et al., 1973). This effect may depend on the state of the luteal cell which is capable of differentiating from one

responsive state to another. In the hypophysectomized rat PRL is unable to initiate the luteotropic response if treatment is delayed for 72 or 96 hours after induction of luteinization (Richards and Williams, 1976). As the luteotropic state is lost an irreversible luteolytic one takes over.

Unlike many other peptide hormones in which target cell response is related to hormone-receptor activation of cAMP, the mechanism by which PRL elicits its action after receptor binding is unknown. Houdebine et al. (1980) suggested that internalization of the hormone-receptor complex (H-R) and lysosomal degradation appears to be unrelated to the mechanism of action of PRL.

1.1 PRL Receptor and Its Regulation

Using antibodies to partially purified PRL receptor, Shiu and Friesen (1976b) demonstrated the importance of the receptor in the biological action of the hormone. The ability of PRL to stimulate casein synthesis in rabbit mammary gland explant (Shiu and Friesen, 1976a) and ovarian activity in the rat (Bohnet et al., 1978) can be inhibited using anti-PRL receptor antibody (Djiane et al., 1981). Furthermore, Djiane et al. (1981) found that at low concentrations the antibody can mimic the actions of PRL in stimulating casein and DNA synthesis. Since PRL receptor antibody can bind to PRL receptor, these results suggest that the hormone does not appear necessary beyond binding to its receptor for transmission of the hormonal response.

Specific receptor sites which interact with lactogenic hormones are localized in a variety of tissues including liver of rats (Ranke et al., 1976), rabbit mammary gland (Posner et al., 1974a; Shiu and Friesen, 1974) rat testicular tissue (Aragona and Friesen, 1975; Charreau et al., 1977) mink uterus (Rose et al., 1983) human chorion-decidua tissue (McWey et al., 1982) and monkey fetal placenta (Josimovich et al., 1977). The number of PRL receptors varies considerably depending on the sex of the animal (Kelly et al., 1974; Kelly et al., 1975) and the physiological state (Djiane and Durand, 1977). The degree of hormonal specificity is tissue dependent (Rose et al., 1983) since in some tissues, PRL receptors are able to bind both lactogenic and somatotropic hormones while in other tissues binding is specific for only one class of hormones.

Luteinized rat ovaries are a rich source of PRL receptors (Koppelman and Dufau, 1982; Harwood et al., 1980a). Apart from the rat, PRL receptors have also been identified in the ovaries of the pig (Rolland et al., 1976) and tammar wallaby (Sernia and Tyndale-Biscoe, 1979). The presence of these receptors in luteinized tissue appears to be functionally related to the ability of luteal cells to respond to PRL (Richards and Williams, 1976). Indeed, modulation of PRL receptors in the ovary during the estrous cycle, including formation and regression of the corpus luteum may account for changes in the intracellular pool of cholesterol and hence in progesterone synthesis.

During LH-induced luteinization receptors for FSH (follicle stimulating hormone), LH and estradiol in both rat (Richards and Midgley, 1976; Richards et al., 1976; Richards and Williams, 1976) and hamster (Oxberry and Greenwald, 1982) decrease while receptors for PRL increase (Richards and Williams, 1976) suggesting that, during this period of luteinization, LH induces an increase in PRL receptors so that the luteal cell becomes more responsive to PRL. On the contrary, Oxberry and Greenwald (1982) using autoradiography found a decrease in PRL receptors after LH-induced luteinization but this loss preceded an increase in receptor numbers.

Using immunocytochemical techniques, PRL receptors have been localized in the ovum as well as in luteal tissue (Dunaif et al., 1982) lending credence to the role of PRL in the maintenance of the corpus luteum and maturation of the ovum. Apart from the ovum, specific receptors for PRL have been identified in non-luteal ovarian tissue in several species (Oxberry and Greenwald, 1982; Midgley, 1972; Richards and Williams, 1976). The function of these sites is unclear, although a possible role for PRL in follicular growth and maturation, in addition to maintaining the functional and structural integrity of the corpus luteum, has been suggested.

Immunocytochemical identification of PRL receptors has not been limited to the cell surface. Bergeron et al. (1978) demonstrated the presence of receptors within specific intracellular structures lending support to the hypothesis that

following binding of the hormone to its receptor, the H-R complex is internalized. In addition, receptor mediated uptake and intracellular translocation of intact ^{125}I-oPRL into Golgi fractions (Josefsburg et al., 1979) and the presence of specific lactogen binding sites for growth hormone (hGH) in the Golgi (Posner et al., 1974b) demonstrate further the ability of polypeptides to move into the cell. These intracellular sites may have several functions including mediation of hormone action and degradation of hormone.

Prolactin receptors are modulated by both homologous and heterologous hormones and have been extensively studied in a wide variety of tissues. It is generally accepted that in the rat several pituitary hormones alone, or in combination (Bohnet et al., 1976) are required to maintain PRL receptors since hypophysectomized females exhibit a loss of hepatic (Posner et al., 1974b) and ovarian (Richards and Williams, 1976) receptors.

Growth hormone may be involved in regulating hepatic PRL receptors since these receptors are induced in rats bearing growth hormone secreting pituitary tumors (Furahashi and Fang, 1978). Furthermore, continuous infusion with hGH induces liver PRL receptors in both gonadectomized-hypophysectomized male and female rats (Norstedt et al., 1981), while rGH stimulates an increase in PRL receptors in hypophysectomized rats. These results suggest that a somatogenic and/or lactogenic hormone is responsible for the induction or up regulation of PRL receptors. However, Norstedt et al. (1981) found

that, although oPRL caused a partial restoration of PRL receptors in hypophysectomized female rats (Costlow et al., 1975), rPRL failed to induce PRL receptors in hypophysectomized rats. Furthermore, previous studies by these authors (Norstedt et al., 1979) found that at the retrochiasmatic level, anterior hypothalamic deafferentation of male rats could still induce an increase in hepatic prolactin receptors without any increase in serum PRL levels. The possibility of GH and not PRL as the primary pituitary factor involved in modulation of liver PRL receptors is further complicated by the studies of Aragona et al. (1976) and Bohnet and Friesen (1976) in which hGH had no effect on PRL binding sites in hypophysectomized male rats and dwarf male mice, respectively. Interestingly, Aragona et al. (1976) found that a combination of ACTH (adrenocorticotropin) and hGH stimulated an increase in PRL receptors.

Modulation of PRL receptors appears to be both hormone and tissue specific. Both FSH (Wang et al., 1979; Navickis et al., 1982) and estrogen (Posner et al., 1974b) stimulate rat PRL receptors in cultured granulosa cells and in the liver, respectively. Similar results were obtained by Richards and Williams (1976) who showed an increase in granulosa cell PRL receptors in FSH-treated hypophysectomized, estrogen primed rats, and by Navickis et al. (1982) who demonstrated an increase in PRL receptors in hCG stimulated-FSH treated rat granulosa cells.

Conversely, treatment of rats with ACTH and LH (Katikineni et al., 1981) caused a loss in PRL binding sites in the adrenal

glands and testis, respectively. A similar decrease was also observed in the rat testis after LHRH/GnRH (luteinizing hormone-releasing hormone) treatment in vivo (Catt et al., 1979a), and in GnRH cultured rat granulosa cells pretreated with FSH to induce PRL receptors (Navickis et al., 1982). The loss of lactogenic binding sites in the gonadal tissue after GnRH treatment of intact rats may be due to a direct action of GnRH on the gonad or may reflect the action of LH released from the pituitary by GnRH.

In immature female rats primed with PMSG and hCG, a loss in luteal PRL receptors occurred after a second injection of hCG given 4 days after hCG-induced luteinization (Davies et al., 1980). The decrease occurred within several hours but was reversible with gradual recovery over the next 2 days.

Finally both estrogen and testosterone caused a loss of PRL receptors in the rat testis (Amit et al., 1983) but co-administration with PRL was unable to restore receptor loss. However, PRL did restore the estradiol-induced decrease in rat prostate PRL receptors.

The reduction in PRL binding sites after stimulation by heterologous hormones involves a mechanism clearly different from that involved in homologous down regulation. In the latter, loss of receptors is due to occupancy which initiates internalization of the hormone-receptor complex as well as unoccupied receptors, followed by processing and/or degradation. However, during heterologous down regulation the decrease in receptors involves a loss of unoccupied sites due to concomitant processing and internalization

of PRL receptors. The formation of a hormone-receptor complex initiated by homologous hormones may stimulate the internalization of unoccupied heterologous sites and so reduce the number of surface heterologous receptors.

Apart from modulation of PRL receptors by heterologous hormones PRL itself appears to regulate its own receptor. This phenomenon has been well documented in both ovarian and testicular tissue with both down regulation and up regulation of PRL receptors depending upon species and tissue specificity. In the testis, PRL down regulates its receptor (Morris and Saxena, 1980; Barkey et al., 1979; Amit et al., 1983). Increasing the concentration of PRL caused a progressive loss of PRL receptors in rabbit mammary gland explant (Djiane et al., 1982) while treatment with bromoergocryptine (CB154) which suppresses PRL secretion, caused either an increase in PRL receptors in rabbit mammary gland *in vivo* (Djiane et al., 1977) or had no effect on PRL receptor levels in rat testis (Huhtaniemi and Catt, 1981b). Djiane et al. (1979a) also demonstrated the loss of PRL receptors, *in vivo*, in both rabbit mammary glands and rat liver after administration of a large dose of PRL. Furthermore, Chan et al. (1981) showed that PRL receptors do not change after treatment with either bPRL or metaclopramide, a dopamine antagonist. These results suggest that PRL is not required in the induction of its receptor. It also appears that microtubules and microfilaments are not involved in down regulation since neither colchicine nor cytochalasin B inhibited this process (Djiane et al.,

1980). The loss of PRL receptors due to homologous hormone involves occupancy of the receptor followed by internalization of the hormone-receptor complex with subsequent lysosomal degradation (Ascoli and Puett, 1978).

Although down regulation of receptors is a common feature of many peptide hormone receptors, several investigators have reported an increase in the number of PRL receptors coincident with high levels of homologous hormone in circulation. PRL treatment increased the specific binding of PRL in both female rat liver membranes (Bohnet and Friesen, 1976; Bohnet et al., 1976; Posner et al., 1975) and rat testicular homogenates (Bohnet and Friesen, 1976). Manni et al. (1978) showed that in hypophysectomized female rats, using purified preparation of PRL, in combination with polyvinyl pyrrolidone to sustain high levels of PRL, could completely restore PRL binding sites in the liver. Furthermore, in dwarf female mice, deficient in pituitary PRL (and GH), transplantation of normal pituitary glands to the kidney capsule restored fertility, suggesting a possible role for the hormone in regulating various reproductive states (Bartke, 1965, 1966). Bohnet and Friesen (1976) also demonstrated a PRL-induced increase in liver PRL receptors in male dwarf mice.

Although up regulation of receptors in response to high levels of homologous hormone is considered to be less common compared to the more frequently reported down regulation observed

with other peptide hormone receptors, several investigators have demonstrated a PRL-induced rapid and reversible down regulation of PRL receptors preceding the well documented increase in receptors. The transient loss of receptors for homologous hormones has been observed both *in vivo* (Djiane et al., 1979a) and *in vitro* (Djiane et al., 1979b, 1980, 1982). This phenomenon has also been reported in the hamster ovary (Oxberry and Greenwald, 1983) prior to heterologous up regulation after LH treatment, and the results appear to be consistent with the loss of PRL receptors coincident with the proestrus gonadotropin (FHS/LH) surge.

The transient loss of PRL receptors in response to homologous hormone may be related to the high turnover rate in target tissue. Djiane et al. (1982) suggested that the high rate of PRL receptor turnover is due to a rapid rate of lysosomal degradation followed by a rapid increase in receptor synthesis. The high turnover rate agrees well with the rapidity of reversibility of the down regulation process.

Whether receptors undergo up or down regulation after exposure to hormones depends on the rate of receptor processing and synthesis. It is possible that the increased rate of PRL receptor synthesis indicated by initial hormone stimulus overrides internalization and degradation of the receptor. It is also possible that the short half life of the PRL receptor and its rapid regeneration (Djiane et al., 1982) may explain why the target cell remains continually sensitive to PRL.

The inconsistent results obtained by many investigators in relation to either up or down regulation may be explained by the differential time points at which receptor numbers were determined (Oxberry and Greenwald, 1983). Within hours of treatment down regulation is expressed whereas between 1 and 2 days after hormone treatment receptor numbers increase to above control values.

As indicated previously, many investigators have observed a loss of receptors after administration of a heterologous hormone, results that are consistent with the more accepted view of receptor loss in the presence of high concentrations of homologous hormone. However, these authors have also demonstrated an irregular transient change in the level of PRL receptors prior to down regulation. Katikineni et al. (1981) reported an early increase in adrenal and testicular PRL receptors after treatment with ACTH and LH, respectively, and that this event preceded the loss of receptors. Similarly, Huhtaniemi et al. (1981) demonstrated a rapid transient increase in both LH and lactogen receptors in rat testicular tissue after treatment with LH which occurred prior to the more recognized loss of receptors.

The rapidity and concomitance of the transient increase in PRL receptors reflects a mechanism that is different to heterologous/homologous up regulation of PRL receptors. Dufau et al. (1978) suggested that such changes are due to exposure of cryptic receptor sites rather than synthesis of new receptors. In addition, Katikineni et al. (1981)

implicated the steroidogenic response in initiating such changes since the transient increase in PRL receptors occurred during the period of active steroid secretion. Increased steroidogenesis may reflect a change in membrane conformation making it more fluid and ultimately exposing hidden receptor sites. Interestingly, Bhattacharya and Vonderhaar (1979) found that methylation of phospholipids in the membrane bilayer increases the fludity of the membrane and enhances hormone binding.

2. Luteinizing Hormone (LH) and Regulation of its Receptor

LH is a glycoprotein composed of an α and β subunit both of which are biologically inactive when isolated but active when recombined.

LH is not required to maintain the function of the rat corpus luteum during the early part of its life span (Richards and Williams, 1976) nor is it required during early development of the corpus luteum in the pregnant rat (Morishige and Rothchild, 1974). The functional role of LH receptor during early luteal cell differentiation is not clear, especially in light of the fact that treatment of primed, immature hypophysectomized rats with LH initiated a decrease, within 24 hours, of LH receptors in the corpus luteum (Richards and Williams, 1976). On the other hand PRL receptor levels increased after LH stimulation and remained elevated in fully luteinized cells.

LH binds to a specific receptor on the plasma membrane to form a hormone-receptor complex which in turn interacts with adenylate cyclase, found on the cytoplasmic portion of the plasma membrane, to catalyze the formation of cAMP. cAMP activates protein kinases and subsequent steroidogenesis.

Coupling of receptors to adenylate cyclase may be enhanced by phospholipid methylation in the cell membrane (Hirata and Axelrod, 1980). Methylation within the luteal cell plasma membrane may be an important mechanism by which stimulatory signals are transmitted (Milvae et al., 1983). The effect of luteotropic hormones may be increased by methylation, stimulating an increase in membrane fluidity and the unmasking of more receptors with subsequent increase in LH binding. The possibility that changes in membrane fluidity increase the chance of the H-R complex interacting with adenylate cyclase should also be considered.

Regulation of the LH receptor is of critical importance in our understanding of the physiological action of LH with respect to the steroidogenic potential of target gonadal tissue. Luteal cells of the ovary and cells of the testis have numerous high affinity and highly specific LH receptors located in the plasma membrane. These receptors are coupled to the adenylate cyclase system. Binding of LH or hCG to the receptors increases both the intracellular and extracellular levels of cAMP with a subsequent increase in progesterone production (Dufau and Catt, 1978).

In both the ovary (Holt et al., 1976) and testis (McNeilly et al., 1979; Belanger et al, 1979), LH receptor appears to be under the major control of PRL. Richards and Williams (1976) showed that, beginning at 48 hours after LH-induced luteinization, exogenous PRL can increase rat luteal LH receptors in vivo, while Grinwich et al. (1976) demonstrated that exogenous PRL prevents LH receptor loss in the same tissue. In the hamster corpus luteum, Harris et al. (1981) also recorded a PRL-induced increase in LH receptors.

In hypophysectomized rats, or animals treated with ergocryptine (CB154), which lowers serum PRL levels, there is a decrease in binding of LH in the ovary (Richards and Williams, 1976; Holt et al., 1976; Behrman et al., 1978) and testis (Aragona et al., 1977; Zipf et al., 1978; Huhtaniemi and Catt, 1981b). Simultaneous treatment of these pituitary PRL deficient animals with PRL restores gonadal LH receptors (Purvis et al., 1979; Aragona et al., 1976). Furthermore, in dwarf mice lacking pituitary PRL, treatment with PRL stimulated an increase in LH receptors (Bohnet and Friesen, 1976) as well as in PRL receptors. Morris and Saxena (1980) also observed an increase in hCG binding in Leydig cells of intact immature rats after an injection of PRL over 2 days, while treatment of male rats with metaclopramide, which induces a rise in PRL secretion, stimulated an increase in LH receptors in testicular tissue (Chan et al., 1981). Huhtaniemi and Catt (1981b) showed PRL-induction of LH receptors under three

separate conditions: (1) during recovery of testicular LH receptors after $GnRH_a$ induced down regulation; (2) during postnatal development of ovarian LH receptors; and (3) during the peripubertal increase in binding of LH to testicular tissue. Interestingly, under the latter condition, hypoprolactinemia delayed the pubertal rise in LH but not PRL receptors.

These results suggest that PRL is necessary for the maintenance of Leydig cell LH receptors (Belanger et al., 1979) in addition to being a prerequisite for the luteotropic action of LH in the luteal cell (Gibori and Richards, 1978), and may reflect a physiological role for PRL in early luteinization through the development of LH receptors. However, it is possible that PRL maintains rather than induces gonadotropin receptors, since Casper and Erickson (1981) found that in rat granulosa cells, cultured for 2-4 days, PRL did not cause an increase in LH receptors although it did prevent a loss in LH receptors. By comparison, however, these authors found that exposing cultured granulosa cells to a high dose of PRL or to PRL that is highly purified caused a marked increase in LH receptors. The difference in results between the studies involving PRL treatment *in vitro* (Casper and Erickson, 1981) and the *in vivo* studies of Richards and Williams (1976) may reflect the length of incubation required to induce LH receptors *in vitro* or may be due to stimulation of LH receptors of the thecal as well as granulosa cells *in vivo*. Furthermore, Morris and

Saxena (1980) suggested a permissive role for PRL whereby PRL modulates the number of LH binding sites.

Apart from PRL, FSH is also involved in heterologous up regulation of LH receptor in the rat testis (Chen et al., 1976) and ovary (Armstring and King, 1971) and in cultured rat granulosa cells (Casper and Erickson, 1981). Similarly PMSG, which has FSH-like activity, increased the activity of the LH-sensitive adenylate cyclase as well as LH receptors in the rat ovary (Salomon et al., 1977). FSH-induced increase in LH receptors may indicate sexual maturation in both male and female rats. It is generally accepted that whereas FSH stimulates adenylate cyclase, with a consequent increase in cAMP, the mechanism of action of PRL is as yet unclear and may not involve the adenylate cyclase/cAMP system. The implication then, that heterologous up regulation of LH receptors after exposure to FSH and PRL may involve several different mechanisms appears to have some foundation.

LH, like many other hormones, modulates its receptor by down regulation in the presence of high concentrations of homologous hormone (Dufau and Catt, 1978; Conti et al., 1976; Conti et al., 1977b; Rajaniemi and Jääskaläïnen, 1979). The decrease in LH receptors after a large or desensitizing dose of hCG may represent an important mechanism in the reduced capacity of gonadotropin stimulable target cell responses. The decrease in LH receptors after gonadotropin injection has been demonstrated in gonadal homogenates (Hsueh et

al, 1976), homogenized ovarian membrane preparations (Conti et al., 1977a), and luteal cells (Conti et al., 1977b) and is initially due to receptor occupancy by the desensitizing hormone which may last up to 24 hours. Prolonged loss of LH binding sites indicates a loss of available surface receptors as a result of internalization. In the rat corpus luteum this prolonged loss may last for as long as 8 days (Conti et al., 1977a).

Although homologous down regulation of LH receptors is well recognized in gonadal tissue, several authors reported a transient increase in LH binding sites preceding the more well recognized down regulation of LH receptors after treatment with hCG or LH (Huhtaniemi et al., 1978, 1981; Suter et al., 1980; Hsueh et al., 1977). This phenomenon may be related to conformational changes in the plasma membrane in which additional surface receptors are unmasked, especially in view of the fact that a concomitant increase in lactogenic receptors was also observed by Huhtaniemi et al. (1978) and Chan et al. (1981). Such changes in the plasma membrane may play an important role in gonadal regulation involving multiple heterologous binding sites. However, positive heterologous receptor regulation reported by Zeleznick et al. (1974) and Zipf et al. (1978) implicate an alternative mechanism in up regulation of surface receptors compared to the more rapid and transitory increase in receptors which precede down regulation.

Contrary to the transient increase in LH receptors after gonadotropin stimulation, Powell et al. (1981) reported a

prolonged up regulation of LH/hCG receptors in the rat testis after hCG injection. Unlike the short period of increase observed by Huhtaniemi et al. (1978), the number of LH receptors increased at 3 hours after treatment and remained elevated for 24 hours before decreasing.

A variety of techniques have been employed to provide evidence for initial binding of hCG/LH to the cell surface. In addition, the loss of hormone bound receptors from the surface of the cell followed by subsequent degradation of the hormone and/or receptor has been well documented to further the concept of receptor-mediated hormonal action.

By using hCG conjugated to horseradish peroxidase, Gulyas et al. (1981) provided a direct visual probe from initial binding of the hormone to the luteal cell surface to cell surface clustering and subsequent internalization of the H-R complex. At $4^{\circ}C$ they observed a random distribution of the plasma membrane-bound conjugate while at $37^{\circ}C$ rapid clustering of the H-R complex was evident. It was also of interest to note that the area adjacent to where clustering and internalization of the hormone conjugate occurred was free of reaction product suggesting an absence of receptors. The implication that binding of hormone to its receptor stimulates an increase in membrane fluidity with consequent movement of receptors to localized areas on the cell membrane prior to internalization should well be considered.

Conn and co-workers (1978), using autoradiography, also demonstrated binding of hCG on the surface of rat luteal cells, in addition to internalization of receptor-bound hCG. Cell surface localization was prominent within 3 hours but by 7 hours internalization of the H-R complex was apparent. Similar techniques have also been employed by Chen et al. (1977) and Han et al. (1974) to show internalization of hCG in the ovary. This phenomenon of internalization of cell surface bound hCG has not only been demonstrated in the rat ovary (Anderson et al., 1979; Conn et al., 1978) but has also been shown to occur in the ovine corpus luteum (Chen et al., 1977) and in cultured rat granulosa cells (Amsterdam et al., 1979).

Internalization of the H-R complex is involved in the process of hormone-induced receptor regulation and also represents an important mechanism by which a cell terminates the stimulatory action of hormones. Furthermore, internalization of hormones is suggestive of a role in hormone mediated cellular activity. The possibility that internalized H-R complex is involved in eliciting cell response was emphasized by Ziminiski et al. (1982) who showed that internalized hCG remains biologically active for a long period of time and may stimulate intracellular activity prior to degradation. Ascoli (1982) showed that through labelling of either the α or β subunit of hCG with ^{125}I, the hormone is internalized intact and reaches its site of degradation in an intact form. The slower rate of internalization of hCG suggests a mechanism that is different compared to the internalization of other proteins.

Degradation of internalized hCG within the luteal cell has been demonstrated by Ahmed et al. (1981) who showed that the half-time for internalization of hCG in ovine luteal cells is approximately 8-12 hours and that the half-time required for internalization and release of radioactive degradative products into the medium was 24 hours. These results are consistent with those of Markkanen and Ranjaniemi (1980), who suggested that internalization and degradation of hCG and its receptor occurred more quickly in the luteal cell compared to the Leydig cell. In addition, direct evidence of degradation of hCG through the appearance of degradative products has been observed by Ascoli and Puett (1978) and Amsterdam et al. (1979). Internalized hCG subunits may be processed at different points and it is possible that subunit dissociation may be involved in the biological actions of hCG (Campbell et al., 1980). Ascoli (1982) further argues that degradation of the hormone may involve processing of one or both subunits of intact hormone followed by subunit dissociation with further degradation of individual subunits. Translocation of internalized gonadotropin and consequent association with lysosomes (Chen et al., 1977; Ascoli and Puett, 1978; Ahmed et al., 1981; Conn et al., 1978) implicated these subcellular organelles with the process of degradation of either the hormone or the hormone and its receptor.

The decrease in gonadal receptors may play a significant role in the reduced hCG/LH stimulated steroidogenic response of target tissue. However, loss of receptors may not be a prerequisite for

either heterologous or homologous desensitization (Hall and Behrman, 1980). In fact, desensitization may precede receptor loss. Caldwell et al. (1980) and Davies and Platzer (1981) suggested that reduced steroidogenesis does not necessitate down regulation of receptors since the period of exposure to gonadotropin necessary to induce a loss of surface receptors is too short. Similarly, degradation of internalized hormone is not required for stimulation of steroidogenesis since Ascoli and Puett (1978) found that NH_4Cl and chloroquine, both lysosomotropic agents, did not effect hormone-stimulated steroid production. Furthermore, Ascoli (1982) demonstrated that cAMP production and steroidogenesis did not require dissociation of hCG, results that were further supported by Ahmed and Niswender (1981).

2.1. LH/hCG-Induced Desensitization

The phenomenon of an attenuated response in terms of cAMP production and steroidogenesis following the administration of LH or hCG has been observed in both rat ovarian and testicular tissues. Several hypotheses have been proposed to explain this hormone-induced loss of biological response including: (1) changes in receptor number and affinity; (2) alterations of coupling between receptor and adenylate cyclase; (3) changes at one or more points along the enzymatic pathway distal to cAMP production; (4) changes in the synthesis of prostaglandins; and (5) an increased rate of cAMP degradation.

Although several authors have suggested a close association between desensitization and the loss of gonadal receptors (Catt et al., 1979b; Anderson et al., 1979) the majority of investigators (Conti et al. 1976; 1977a), at present, point to the loss of adenylate cyclase activity as the mechanism involved in reduced steroidogenesis during desensitization.

hCG-induced heterologous and homologous desensitization have been demonstrated in the rat (Hunzicker-Dunn et al., 1979; Harwood et al. 1980b). Bockeart et al. (1976) demonstrated a loss in ovarian adenylate cyclase activity after hCG treatment while Hunzicker-Dunn and Birnbaumer (1976) have shown that in the rat graafian follicle, the preovulatory surge of gonadotropin promotes desensitization *in vivo*, of the LH-sensitive adenylate cyclase. Similar results have been shown *in vitro* by Hunzicker-Dunn and Birnbaumer (1981) and Lamprecht et al. (1973) using rabbit and rat graafian follicles, respectively. Marsh and co-workers (1973) also found that rabbit preovulatory follicles do not respond to LH, *in vitro*, after either an *in vivo* injection of hCG or after the endogenous surge of LH.

It is of interest to note that although Anderson et al. (1979) found a close correlation between receptor loss and reduced responsiveness of adenylate cyclase to LH, they also present the possibility of inactivation of the catalytic site of adenylate cyclase, particularly in view of the partial refractoriness of adenylate cyclase to fluoride stimulation.

Apart from loss of activity in the catalytic subunit, a reduction in adenylate cyclase activity is also suggestive of a defect in the coupling of receptor to adenylate cyclase. Harwood et al. (1980b) have shown a decrease in progesterone and cAMP production in gonadotropin-induced desensitized luteal tissue after LH and epinephrine stimulation. Although LH receptors decreased, receptors for epinephrine remained unchanged, indicating that the loss in steroidogenesis and cAMP production after epinephrine stimulation may result from a possible defect in the coupling of β-adrenergic receptors to adenylate cyclase due to gonadotropin desensitization. Similar results have been obtained by Abramowitz and Birnbaumer (1982) who found that during heterologous desensitization, LH and isoproteranol act via the same adenylate cyclase system.

The phenomenon of rapid homologous desensitization involves uncoupling of the H-R complex from the N (nucleotide) regulatory subunit of adenylate cyclase without an alteration in the N component. (Kirchick et al., 1983). Uncoupling from the adenylate cyclase system is probably due to an alteration (phosphorylation?) of the receptor since homologous desensitization is hormone specific. Phosphorylation of the receptor may target the cell for internalization. Although this mechanism may explain the initial loss of LH stimulable adenylate cyclase activity in hCG desensitized rat ovaries, longer periods of desensitization may involve down regulation of receptors in addition to receptor alteration.

On the other hand, heterologous desensitization may necessitate an alteration in the coupling protein without an effect on receptor, suggesting that, not unlike the conclusions of Abramowitz and Birnbaumer (1982), there is no distinction between the N component of the LH-adenylate cyclase and the β-adrenergic-adenylate cyclase systems.

In cultured rat luteal cells obtained from hCG desensitized rats, LH/hCG was unable to stimulate cAMP or progesterone production (Conti et al., 1977b) with the possible implication that luteal cell desensitization may be the result of a defect in the pathway of progesterone production distal to cAMP production. In addition, these authors demonstrated that progesterone production returned to normal levels well before restoration of LH receptors or cAMP production, indicating that the maintenance of steroidogenesis may be dependent on endogenous gonadotropins acting on few, albeit adequate, receptor numbers. Similar results have been reported by Davies and Platzer (1981) using rat Leydig cells. The implication of a post receptor block in testosterone production after hCG-induced loss of testicular responsiveness was suggested in view of the fact that cAMP given after hormone-induced desensitization did not restore steroid levels. Furthermore, the rapidity of loss of steroidogenesis compared to the longer time taken for LH receptors to down regulate suggests that down regulation is not involved in the reduction in steroidogenesis.

cAMP may be an important mediator of the desensitizing process. However, results from previous investigators have shown variation

from a total lack of influence of cAMP on adenylate cyclase desensitization (Lefkowitz et al., 1980) to a lack of responsiveness of adenylate cyclase to LH stimulation in the cAMP primed preovulatory rabbit follicle (Hunzicker-Dunn and Birnbaumer, 1981). Hedin and Ahrēn (1983) also showed a partial desensitization of the prepubertal rat ovary to LH stimulation after treatment with cAMP analogues. Both Hedin and Ahrēn (1983) and Hunzicker-Dunn and Birnbaumer (1981) hypothesized that LH-induced desensitization involves an initial phase not mediated by cAMP followed by a cAMP mediated secondary phase. The latter phase may involve inhibitory proteins or phosphorylated membrane proteins which alter adenylate cyclase or inhibit coupling between receptor and adenylate cyclase.

It is of interest to note the possibility of a relationship between desensitization and luteolysis. Both mechanisms cause a loss in LH receptors accompanied by a decrease in LH-stimulable cAMP and progesterone production. Since changes observed after $PGF_{2\alpha}$-induced luteolysis are not reversible while changes due to gonadotropin-induced desensitization are reversible, Conti et al. (1977a) hypothesized that desensitization of the ovary may be a transient form of "reversible luteolysis."

3. Coordinate Regulation of LH and PRL Receptors

Down regulation of receptors from the surface of gonadal cells is seen as a mechanism designed to reduce target cell sensitivity to continued or excessive hormonal stimulation.

The effects of a variety of hormones on the function of the corpus luteum emphasize the complexity of multiple receptor regulation for the luteotropic hormones, LH and PRL. Several investigators have reported concomitant changes in both LH and PRL receptors in gonadal tissue after hormonal stimulation. This phenomenon is of particular interest in the study of luteal function since it is known that both LH and PRL receptors are required for the functional maintenance of the corpus luteum and that changes in the number of one type of receptor during luteal formation or regression may predispose changes in the other type.

Coordinate regulation of LH and PRL receptors has been demonstrated mainly in studies involving testicular tissue with lesser emphasis on luteotropic hormone receptor regulation in the ovary. Treatment of rat testicular tissue, in vivo, with ovine LH caused an increase in LH and PRL receptors within 40-120 minutes (Chan et al., 1981). The initial rise in receptors was followed by a transient loss and recovery of luteotropic binding sites. The negative effect of LH on PRL receptors was due to a true loss of receptors and not due to receptor occupancy since radiommunoassay for serum PRL showed no increase after gonadotropin treatment (Davies et al., 1980). Recovery of PRL receptors occurred within 48 hours compared to a more sustained loss of LH receptors. This may reflect a faster turnover rate for PRL receptors. The concomitant loss of LH and PRL receptors following a transient increase and their subsequent differential rates of recovery

in testicular tissue after gonadotropin treatment have been shown in similar studies by Davies et al. (1980), Auclair et al. (1977a), and Chan et al. (1981). These observations suggest that the two receptor sites may be physically related within the target cell membrane (Davies et al., 1980), although nonspecific cointernalization of free lactogen receptors with LH binding sites cannot be overruled.

In immature rats, primed with PMSG and hCG to induce luteinization of ovaries, a rapid fall in LH and PRL receptors was demonstrated after a second dose of hCG (Davies et al., 1980). Although the decrease in receptors occurred earlier than in the testis, lactogen receptors returned to normal at 48 hours after hCG treatment, whereas the number of LH receptors was still lower by 3 days, comparable to values obtained at 4 days in the testis (Davies et al., 1980). It is of interest that, in the experiments of Davies and co-workers (1980), there was no change in luteal receptors for insulin and β-adrenergic ligands indicating that the loss of PRL receptors, after heterologous hormone treatment, is not just a generalized effect on cell membrane receptor turnover. Katikineni et al. (1981) and Davies et al. (1980) suggested that the mechanism of heterologous loss of lactogen receptors involves a specific clustering and co-internalization of both LH and PRL sites.

A similar loss in luteotropic receptor sites has been demonstrated in testicular tissue after treatment with Gonadotropin Releasing Hormone (GnRH). A single dose of GnRH caused a marked

transitory loss of FSH, LH and PRL receptors in the rat testis (Catt et al., 1979a). In this study, the loss of LH receptors was maximal 2 days after the LH surge. The LH peak, presumably caused by the action of GnRH on the pituitary, may be involved in down regulation of LH receptors with a subsequent loss in heterologous receptor. Furthermore, down regulation of LH receptors by GnRH agonists has also been explained by a GnRH agonist-induced pituitary LH surge (Rippel and Johnson, 1976). However, the possibility of a direct action of GnRH on the gonad cannot be overlooked especially in light of the presence of specific binding sites for GnRH on gonadal tissue. Similar changes in LH and PRL binding sites in the testis after treatment with GnRH analogues have been observed by Huhtaniemi and Catt (1981b) and Auclair et al. (1977a).

Interestingly, a report by Sandow and Robyn (1972), in which they observed a transient fall in serum PRL after GnRH treatment in male rats, suggests that GnRH may act as a dopamine agent. The decrease in PRL may cause a loss of PRL receptors since PRL is necessary for the induction of its receptor. In addition this change in serum PRL level may also cause a reduction in LH receptors since both GH and PRL have been shown to be involved in the induction and maintenance of testicular LH receptors (Payne and Zipf, 1978).

In summary, the common location of testicular/ovarian LH and PRL receptors and the similarities in loss after LH/hCG or GnRH treatment imply that down regulation after gonadotropin stimulation is coodinated and suggests a physical relationship between the two

receptors. However, the differential rates of recovery indicate an element of independence.

4. $PGF_{2\alpha}$ and Luteal Regression

In sexually immature, superovulated rats the plasma levels of progesterone decrease from around day 9 of the life span of the corpus luteum. The reduced functional activity of this endocrine gland and the subsequent involution of luteal cells is referred to as luteolysis.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is widely regarded as the physiological luteoysin, inducing regression of the corpus luteum in many species including the rat (Pharris et al., 1972; Horton and Poyser, 1976; Chang and Hunt, 1972). Its effects appear to be indistinguishable from those reported during spontaneous luteolysis in terms of the levels of plasma progesterone and 20_{α}-dihydroprogesterone (20_{α}-DHP) and the number of ovarian LH binding sites.

In most mammals $PGF_{2\alpha}$ appears to be of uterine origin since hysterectomy prolonged the functional life span of the corpus luteum (Horton and Poyser, 1976; Anderson et al., 1969). Horton and Poyser (1976) also showed that $PGF_{2\alpha}$ induces luteal regression in the hysterectomized gilt. In addition, indomethacin, which inhibits prostaglandin synthesis, prevented luteolysis at the end of pregnancy in swine (Nara and First, 1977) and prolonged psuedopregnancy in rabbits (Carlson and Gole, 1978). Similar results have been achieved

using the prostaglandin synthetase inhibitor BPPA (2,p-biphenyl propionic acid) (Cochrane et al., 1983) with the implication that the inhibitor acts by means of an antiluteolytic rather than a luteotropic mechanism. It is also of some interest that endogenous luteal prostaglandins may be involved in refractoriness induced by LH treatment (Evrard-Herouard et al., 1981) especially since Demers and coworkers (1973) demonstrated LH stimulation of prostaglandin synthesis in rat corpora lutea. However, Kirchick and Birnbaumer (1981) argue against this hypothesis since indomethacin did not prevent hCG-induced desensitization in rabbit corpora lutea.

$PGF_{2\alpha}$-induced luteolysis of the superluteinized rat is characterized by a rapid drop in serum progesterone within 30 hours of treatment (Grinwich et al., 1976). Administration of the $PGF_{2\alpha}$ analogue, cloprostenol, to these rats caused an immediate (within 25 minutes) decrease in serum progesterone (Torjesen et al., 1978a), followed by an increase in PRL levels 3-6 hours later. Serum levels of 20α-DHP rose within 6-9 hours after $PGF_{2\alpha}$ treatment. These authors (Torjesen et al., 1978a,b) suggested that PRL is necessary to stimulate 20α-hydroxysteroid dehydrogenase activity in luteal tissue with a subsequent increase in 20α-DHP. Furthermore, Torjesen et al. (1978a) proposed that low levels of progesterone initiate an increase in PRL secretion, which is in agreement with the results of Haug and Gautvik (1976) who showed that high levels of progesterone inhibit secretion of PRL by rat pituitary cells in culture. However, the possibility of direct stimulation of pituitary PRL secretion

by $PGF_{2\alpha}$ cannot be overlooked, particularly since Gautvik and Kriz (1976) reported a $PGF_{2\alpha}$-induced stimulation of PRL secretion in rat pituitary cells in vitro while Burton et al. (1975) observed a similar result in intact male rats.

In association with these changes in plasma steroid levels, the number of LH/hCG binding sites is severely reduced after $PGF_{2\alpha}$ treatment. This may imply that regression is due to an induced loss in the ability of the corpus luteum to respond to gonadotropin. $PGF_{2\alpha}$, in vitro (Behrman et al., 1974), and in vivo (Behrman et al., 1971), caused a marked loss in the ability of luteal cells to bind gonadotropins in vivo (Hichens et al., 1974). The effects of $PGF_{2\alpha}$-induced reduction in plasma progesterone and LH binding sites in the corpus luteum are also observed during the latter half of pseudopregnancy in the superluteinized rat (Lee and Ryan, 1973; Torjesen and Aakvaag, 1977).

Coincident with the decrease in ^{125}I-hCG binding to luteal tissue, Behrman and co-workers (1978) found that $PGF_{2\alpha}$ also caused a reduction in binding of ^{125}I-oPRL. Interestingly, these authors also showed that intact rats treated with PRL, prior to administration of $PGF_{2\alpha}$, did not exhibit a decrease in either ^{125}I-hCG binding to luteal cells or serum progesterone levels. The possibility that PRL regulates LH receptors, thereby controlling the sensitivity of the corpus luteum to the luteolytic action of $PGF_{2\alpha}$, is well documented and has been discussed earlier in this survey.

Unlike hCG-induced desensitization of gonadal tissue, the loss of LH binding sites after $PGF_{2\alpha}$ treatment is irreversible. Part of this loss may be associated with cellular disruption involving destruction of luteal tissue (Stacy et al., 1976). Again, in terms of morphological and biochemical evidence (McClellan et al., 1977) $PGF_{2\alpha}$-induced luteal regression is very similar to spontaneous regression.

The mechanism of action of $PGF_{2\alpha}$ is, as yet, unclear but may involve either a direct or indirect effect on the corpus luteum or a combination of both. Several hypotheses have been postulated to explain the indirect effect of the luteolytic action of $PGF_{2\alpha}$:

1. $PGF_{2\alpha}$ decreases blood flow to the ovary of corpus luteum (Niswender et al., 1976; Novy and Cook, 1973; Niswender et al., 1975). However, Behrman et al. (1971) found that serum progesterone levels decrease well before any observable change in blood flow to the rat ovary. Further arguments for and against an effect by $PGF_{2\alpha}$ on blood flow have been provided by Behrman et al. (1978) who indicated a permissive role for PRL in $PGF_{2\alpha}$-induced changes in blood flow. It is of interest to note that Pang and Behrman (1981) observed a transient increase in blood flow to ovarian interstitial tissue but that this was not due to any decrease in blood flow to the corpus luteum. They suggested a regional vasodilatory effect of $PGF_{2\alpha}$ which may be involved in stimulating follicle development after induction of luteal regression. Furthermore, these authors (1979) also established that progesterone levels

in rats decreased toward the end of pseudopregnancy before any significant reduction in blood flow, and that there was no change in blood flow to the corpora lutea of pseudopregnant rats treated with $PGF_{2\alpha}$ (Pang and Behrman, 1979).

2. $PGF_{2\alpha}$ inhibits maintenance of luteal function by blocking the uptake of gonadotropin *in vivo*, rather than necessitating a loss in LH receptors (Behrman and Hichens, 1976). Similarly, Pang and Behrman (1981) also observed a rapid block of gonadotropin uptake by the corpus luteum *in vivo*, occurring within one hour of $PGF_{2\alpha}$ treatment in pseudopregnant rats. The rapid suppression of progesterone secretion was independent of either a change in luteal blood flow or the number of LH receptors. Blood supply was eventually reduced 36 hours after $PGF_{2\alpha}$ treatment, but this effect was not due to a direct effect by $PGF_{2\alpha}$. However, further studies by these authors, in which $PGF_{2\alpha}$ did not directly reduce gonadotropin binding in isolated luteal cells, indicate that $PGF_{2\alpha}$-dependent inhibition of gonadotropin uptake, *in vivo*, is due to the inability of luteal cells to receive gonadotropin. The restricted access to LH may be the result of a decrease in capillary permeability or impedence of diffusion of gonadotropin across the interstitium.

3. Although it is generally agreed upon that $PGF_{2\alpha}$-induced regression of the corpus luteum is independent of the pituitary, several investigators have reported an increase in pituitary LH release after treatment of breeding (Carlson et al., 1973) and

immature (Bono et al., 1980) ewes with $PGF_{2\alpha}$. In estrogen pre-treated estrus rabbits both PGE_2 and $PGF_{2\alpha}$ stimulated LH release with the latter being a more potent luteolytic agent (Carlson et al., 1977). In the rat, Warberg et al. (1976) reported an increase in the release of pituitary LH as a result of $PGF_{2\alpha}$ treatment, although Harms and co-workers (1974) demonstrated that PGE_2 was more effective than $PGF_{2\alpha}$ in stimulating LH release. However, in a quantitative study on LH receptors in the ewe (Diekman et al., 1978b) the reduction in hCG binding was not the result of increased occupancy of LH receptors by $PGF_{2\alpha}$-stimulated endogenous LH. Several reports suggest that prostaglandins stimulate LH release through the hypothalamus or pituitary (Bono et al., 1980; Carlson et al., 1977). However, Carlson et al. (1973) hypothesized that changes in LH are due to alterations in hormones secreted by the ovary after treatment with $PGF_{2\alpha}$, rather than to a direct effect of $PGF_{2\alpha}$ on the hypothalamo-pituitary axis. On the other hand, Hafs (1975) demonstrated that the hypothalamus is the principal target organ in the release of PRL by prostaglandins.

The presence of high affinity binding sites for $PGF_{2\alpha}$ on the rat corpus luteum (Wright et al., 1979) and the fact that the corpus luteum is refractory to $PGF_{2\alpha}$-induced luteolysis on or before day 4 of pregnancy or pseudopregnancy suggests a direct effect of $PGF_{2\alpha}$ on luteal regression which is receptor-mediated. Evidence for a direct effect has been provided by Behrman et al. (1974) who demonstrated that $PGF_{2\alpha}$ blocks LH stimulation of progesterone in

cultured rat luteal cells. $PGF_{2\alpha}$ may cause regression by regulating the number of LH binding sites on the ovary especially since many studies have reported a decrease in LH binding sites along with a reduction in progesterone levels (Hichens et al., 1974). Behrman et al. (1978) postulated that the loss of LH receptors induced by $PGF_{2\alpha}$ might be the stimulus in initiating luteolysis with subsequent alterations in progesterone and 20_{α}-DHP levels. However, several reports (Grinwich et al., 1976; Torjesen et al., 1978a) showed a decrease in serum progesterone preceding the loss of LH receptors in the immature, superovulated rat. Similar results were obtained by Diekman et al (1978b) using the estrus ewe as the model. The study by Garris et al. (1980) demonstrated a decrease in progesterone production prior to a reduction in LH receptors after treatment of rats with antibodies to LH. A concomitant reduction in PRL levels suggests that the loss in LH receptors is a function of reduced serum PRL. This change in PRL levels after $PGF_{2\alpha}$ treatment is, however, contrary to the results obtained by Torgesen et al. (1978a, b) using the $PGF_{2\alpha}$ analogue, cloprostenol.

A direct effect of $PGF_{2\alpha}$ on the luteal cell membrane *in vitro*, and, *in vivo*, may involve inhibition of LH stimulable adenylate cyclase activity (Thomas et al., 1978; Spicer et al., 1981; Henderson et al., 1977) and cAMP production (Lahav et al., 1976). These events may be more important, compared to a loss of LH receptors, in reducing the functional activity of the corpus luteum during regression. However, Jordan (1981) found that dibutyryl cAMP

did not stimulate steroidogenesis in $PGF_{2\alpha}$ treated dispersed luteal cells and suggested that $PGF_{2\alpha}$ acts on a site distal to the generation of cAMP (Cigorraga et al., 1978).

It is also of interest to note that between days 4 and 10 of pseudopregnancy in the rat, Wright et al. (1980) found no change in the affinity or capacity of $PGF_{2\alpha}$ receptors with the implication that increased sensitivity to $PGF_{2\alpha}$-induced luteolysis during the latter half of pseudopregnancy is not due to a change in receptor number of affinity. This is in direct contrast to the increase in $PGF_{2\alpha}$ receptors towards the end of pseudopregnancy reported by Fitz and coworkers (1982).

Finally, several reports have indicated that phase changes in the phospholipid bilayer of luteal cells may be involved in luteal regression. Binding of $PGF_{2\alpha}$ to specific luteal receptors may induce lipid phase changes in the phospholipid bilayer of cell membranes causing a decrease in the structural integrity of the cell. The presence of increasing gel phase in luteal membranes following $PGF_{2\alpha}$ treatment causes a loss in luteal function (Carlson et al., 1982). In senescing bean plants McKersie and Thompson (1977) demonstrated an increase in gel phase lipid, paralleling a rise in the lipid phase transition temperature of cell membranes. The presence of a mixture of gel and crystalline lipid phases may cause membranes to become leaky (Papahadjopoulos et al., 1973). A similar mixture of lipid phases, accompanied by an increase in the lipid phase transition temperature, has also been observed in microsomal membrane preparations from animals undergoing spontaneous regression or $PGF_{2\alpha}$-induced

regression (Buhr et al., 1979) suggesting that the mechanism of luteal regression may involve a phase change in the lipid bilayer.

5. Gonadotropin Releasing Hormone (GnRH)

GnRH is a decapeptide produced and secreted by the hypothalamus. Under physiological conditions GnRH stimulates pituitary secretions of LH and FSH. However, pharmacological doses of GnRH have been shown to inhibit the release of gonadotropins from the pituitary gland, a phenomenon of recent importance in clinical applications involving contraception.

GnRH exerts both inhibitory (Kledzik et al., 1978a, b; Beattie and Corbin, 1977) and stimulatory (Clarke et al., 1980) effects on ovarian function.

The antifertility effects of GnRH and its analogs include inhibition of hCG-induced increase in ovarian weight (Rippel and Johnson, 1976), a decrease in progesterone and estrogen production in isolated rat luteal cells (Hsueh and Erickson, 1979a; Clayton et al., 1979), a loss of LH stimulation of luteal cells (Clayton et al., 1979; Behrman et al., 1980; Harwood et al., 1980d) and inhibition of FSH-induced follicular development (Ying and Guillemin, 1979) and granulosa cell differentiation (Hsueh et al., 1980; Knecht et al., 1981; Hsueh and Ling, 1979). GnRH may also be involved in luteolysis (Bex and Corbin, 1979; Casper and Yen, 1979), particularly since McDonald and Beattie (1979) reported the failure of pregnancy in hypophysectomized rats treated with GnRH. Furthermore,

Pieper et al. (1980) characterized the role of GnRH in luteolysis as a decrease in steroidogenesis concomitant with an increase in receptors for GnRH on the luteal cell surface. Apart from the ovary, GnRH has also been shown to exert an inhibitory effect on testicular tissue involving a loss of PRL and LH receptors and reduced testosterone production (Catt et al., 1979a).

Several hypotheses have been developed to explain the anti-gonadal effects of GnRH:

1. Continuous exposure or high doses of GnRH may result in pituitary refractoriness with a consequent decrease in gonadotropin output (De Koning et al., 1978; Belchetz et al., 1978). Clayton (1982) demonstrated an up regulation of GnRH receptors in the pituitary after continuous infusion of a low dose of GnRH. The increase in pituitary GnRH receptors after GnRH stimulation has also been observed by others (Frager et al., 1981) and did not result in desensitization of pituitary gonadotropin secretion. On the other hand, continuous high doses of GnRH caused desensitization of the pituitary gland in releasing gonadotropins. This may result from either a loss of GnRH receptors, depletion of pituitary LH stores, or a combination of both (Clayton, 1982). However, the role of GnRH receptor down regulation in the mechanism of desensitization is not clear. Although some investigators have shown a correlation between receptor loss and pituitary refractoriness to GnRH, in vivo (Clayton, 1982), others have not been able to demonstrate a loss of pituitary GnRH receptors, although desensitization to GnRH had occurred (Sandow et al., 1979).

2. GnRH may exert an indirect inhibitory effect on the gonad by stimulating the release of large amounts of LH and FSH (Schally, 1978) resulting in secondary desensitization of gonadal tissue. The elevation of GnRH-induced serum gonadotropins and the resultant down regulation of gonadal receptors and reduced steroidogenesis (Catt et al., 1979a; Cusan et al., 1979; Sharpe et al., 1979) have been well documented (Auclair et al., 1977a, b; Kledzik et al., 1978a, b) and may explain both the inhibitory as well as stimulatory roles of GnRH (Naor et al., 1983) on gonadal tissue. Intact male rats treated with a GnRH agonist exhibited a loss in gonadotropin receptors and inhibition of both steroidogenesis and spermatogenesis (Seguin et al., 1981). The loss in testicular LH receptors was not due to a direct effect of GnRH on the testis but was initially due to high levels of LH secreted from the pituitary followed possibly by a long term effect involving pituitary desensitization to continued stimulation. Similarly, studies by Reddy et al. (1980) demonstrated peak levels of LH occurring one hour after treatment of female rats with GnRH suggesting that ovarian down regulation of LH receptors after GnRH stimulation is a consequence of a transient LH surge. The subsequent reduction in ovarian steroidogenesis reflects a loss in both LH receptors and cAMP production.

3. Several studies involving hypophysectomized rats demonstrated a direct effect of GnRH on the ovary (Rippel and Johnson, 1976; Bex and Corbin, 1979; Hsueh and Erickson, 1979a). GnRH inhibits PRL maintenance of luteal function in hypophysectomized

female rats, in vivo (Jones and Hsueh, 1980). In addition, using the the same model, Hsueh and Erickson (1979a) demonstrated a reduction in FSH stimulation of estrogen production in the ovary while Jones and Hsueh (1981a) showed that concomitant treatment with a GnRH antagonist blocked GnRH inhibition of FSH-induced increases in ovarian weight, hCG binding and steroidogenesis in the hypophysectomized rat. Both Seguin et al. (1981) and Harwood et al. (1980d) observed a decrease in ovarian LH receptors after administration of GnRH to intact and hypophysectomized immature female rats primed with PMSG/hCG. In addition, a transient loss in PRL receptors along with reduced progesterone production was also observed (Harwood et al., 1980d). The reduction in both LH and PRL receptors after GnRH stimulation paralleled a loss in receptors observed after hCG stimulation. Similar observations have been reported by Clayton et al. (1980) who demonstrated a loss of LH and PRL receptors in the testis of hypophysectomized rats after treatment with LH or GnRH. Although initial PRL receptor loss was similar after either treatment a more prolonged effect after GnRH treatment suggests that LH and GnRH regulate gonadal PRL receptors through different mechanisms. Interestingly, since hCG/LH stimulates steroid production in gonadal tissue of hypophysectomized rats, Harwood et al. (1980d) speculated a role of hCG-induced PRL receptor loss involving the uptake of precursors for steroid production. On the other hand, the effects of GnRH on PRL receptors may not be related to steroidogenesis since GnRH decreases steroid levels in hypophysectomized

rats. Further observations of a direct inhibitory effect by GnRH on testicular tissue of hypophysectomized male rats have been reported by Hsueh and Erickson (1979b) and Bambino et al. (1980). These results suggest an extrapituitary, direct gonadal site of action of GnRH is more important than a pituitary effect, although, in intact adult animals, the release of endogenous LH may also contribute to the loss of gonadal LH and/or PRL receptors and subsequent inhibition of gonadal responsiveness to GnRH.

Additional evidence for an extrapituitary action by GnRH has been provided through several in vitro studies. GnRH inhibits steroidogenesis in isolated granulosa and luteal cells (Hsueh and Erickson, 1979a; Hsueh et al., 1980; Clayton et al., 1979) and in long term Leydig cell cultures (Hsueh and Erickson, 1979b). Furthermore, concomitant treatment with a GnRH antagonist blocked GnRH-inhibition of FSH, PRL- and cholera toxin-stimulated steroidogenesis, in vitro (Jones and Hsueh, 1981a). Since GnRH blocked the FSH-induced increase in estrogen production, in vitro, Hsueh and Erickson (1979a) hypothesized that inhibition of extrogen-dependent processes such as ovulation, ovum transport and pregnancy is due to a decrease in FSH-stimulated estrogen production by GnRH. Similarly the decrease in progesterone, in vitro, may reflect the mechanism of action of GnRH in blocking pregnancy.

Finally, identification of specific, high affinity binding sites for GnRH in the rat luteal cell (Clayton et al., 1979; Harwood et al., 1980c) and ovary (Heber et al., 1978; Pieper et al.,

1980; Perrin et al., 1980) and the uptake of ^{125}I-GnRH analogue in the rat ovary (Mayer et al., 1979) suggests that GnRH does not exert its effect solely at the level of the pituitary and that the anti-fertility effects of GnRH are mediated by binding to specific GnRH receptors on the surface of gonadal cells (Reeves et al., 1980). These receptors appear to have similar binding characteristics to receptors in the pituitary and are found in immature PMSG/hCG primed rats containing granulosa and luteal cells. However, the uncertainty as to a physiological action of GnRH is apparent when one considers the extremely minute quantities of GnRH reported in the portal blood system of the rat (Eskay et al., 1975) coupled with the high dilution of the hormone by the time it reaches the ovary. Even though the amount of GnRH reaching the ovary from the pituitary is very small, several investigators (Reeves et al., 1980; Ying and Guilleman, 1979; Pieper et al., 1980) have argued for a physiological role for these receptors and have implicated the action of local GnRH-like peptides (gonadocrinins) in the inhibition of gonadal function. Similar compounds may also be present in the testis (Bourne et al., 1982) since receptors for GnRH have also been localized in this tissue (Bourne et al., 1980; Clayton et al., 1980).

The regulation of gonadal GnRH receptors is under the control of its homologous hormone. GnRH caused an up regulation of its receptor in the testis of both intact and hypophysectomized rats (Bourne et al., 1982) similar to the increase in GnRH receptors observed in the pituitary (Frager et al., 1981) after GnRH

stimulation. Homologous up regulation of receptors has also been observed with PRL (Posner et al., 1975) and angiotensin II (Hauger et al., 1978). In the rat corpus luteum, Pieper et al. (1980) reported an increase in GnRH receptors following injection of a GnRH analogue into pregnant rats. This was not observed either in the corpora lutea or the ovary of cycling rats suggesting that GnRH is more effective in potentiating steroidogenically active tissue such as the corpus luteum of pregnant rats. Interestingly, White and Ojeda (1981) demonstrated a marked loss in ovarian GnRH receptors associated with activation of steroidogenesis which precedes the first gonadotropin surge in prepubertal rats. They suggested a decrease in GnRH receptors reduces the inhibitory function of GnRH in the ovary so that at puberty, under the influence of gonadotropins, the production of steroids by the ovary occurs at a more rapid rate.

The mechanism by which GnRH exerts a direct desensitizing effect, in vitro, and, in vivo, is still uncertain although reports by several investigators lead one to suspect that GnRH may act at several different points along the steroidogenic pathway. GnRH inhibits epinephrine and hCG stimulation of progesterone production in isolated rat luteal cells indicating an effect at a post-receptor locus (Clayton et al., 1979; Harwood et al., 1980c). However, the loss of FSH receptors or impaired coupling between receptor and adenylate cyclase may be involved in GnRH inhibition of FSH and LH dependent adenylate cyclase, in vivo (Ranta et al., 1983). The addition of dibutyryl-cAMP prevented the action of GnRH in inhibiting progesterone production in cultured rat luteal cells indicating that

the mechanism of GnRH inhibition involves a reduction in adenylate cyclase activity (Behrman et al., 1980). In addition GnRH did not inhibit binding of ^{125}I-hCG to luteal cells reflecting a mechanism of action that involves binding to its receptor on the luteal membrane with a possible impairment in the functional coupling between receptor and adenylate cyclase. Similar results by Harwood et al. (1980c) demonstrated that GnRH had no effect on dibutyryl- cAMP stimulation of progesterone production in luteal cells and furthermore did not affect the affinity or capacity of LH binding sites. On the contrary, a post cAMP site of action for GnRH has been suggested (Reddy et al., 1980) in view of the fact that GnRH did not inhibit cAMP accumulation in response to hCG or cholera toxin. This post cAMP site of action for GnRH may involve inhibition of 3β-hydroxysteroid dehydrogenase, (3β-HSD), the enzyme required to convert pregnenolone to progesterone, or the stimulation of 20α-hydroxysteroid dehydrogenase, the progesterone metabolizing enzyme (Jones and Hsueh, 1981b, 1982). Alteration in adenylate cyclase activity or sites distal to cAMP production may involve binding of GnRH to its receptor followed by stimulation of phosphatidylinositol and phosphatidic acid. These changes may act as a transducing mechanism for the effect of GnRH in luteal and pituitary cells (Leung et al., 1983; Snyder and Bleasdale, 1982).

The actions of GnRH are comparable to the effects of $PGF_{2\alpha}$ induced luteolysis in terms of a decrease in LH receptors and steroidogenesis. Behrman et al. (1980) demonstrated that GnRH

does not affect $PGF_{2\alpha}$ receptors but both may exert their similar effects through a common mediator. It is also interesting to reflect on the apparent similarities between the action of GnRH and LH with respect to prostaglandin formation in the ovary, oocyte maturation and ovulation in the hypophysectomized rat. Dekel et al. (1983) showed that GnRH and LH do not share a common receptor in the ovary but the similarities in their stimulatory effects implicate a common mechanism at a site distal to the receptor.

CHAPTER II

THE EFFECT OF hCG, GnRH and $PGF_{2\alpha}$ ON SERUM PROGESTERONE, OVARIAN LH AND PRL RECEPTORS AND MORPHOLOGY OF THE CORPUS LUTEUM IN SUPEROVULATED RATS

1. Introduction

It is well recognized that the mechanism of action of peptide hormones involves initial selective binding to specific receptors located on the plasma membrane resulting in the formation of a hormone-receptor complex and activation of the adenylate cyclase system (Catt and Dufau, 1976; 1977). The subsequent increase in intracellular cAMP levels mediates a variety of hormonally-induced physiological responses (Dufau and Catt, 1978).

However, continued stimulation with high levels of hormones causes desensitization in which cells are unable to respond in terms of an increase in steroidogenic activity. The mechanism of desensitization is, as yet, unclear but may involve a decrease or down regulation in the number of available receptor sites (Conti et al., 1977a) so that the sensitivity of the target cell to homologous hormone is reduced (Tsuruhara et al., 1977; Catt et al., 1979b). In the gonad the loss of receptors is believed to occur by internalization of the hormone-receptor complex followed by lysosomal degradation of the hormone and possibly the receptor (Chen et al., 1977; Amsterdam et al., 1979). Recycling of dissociated intracellular receptors back to the cell surface or intracellular synthesis of new receptors and subsequent

translocation to the plasma membrane restores the transient, hormone-induced loss of surface receptors.

Furthermore, a concomitant decrease in the number of receptors for hormones unrelated to the primary ligand has been demonstrated in ovarian and testicular tissue (Chan et al., 1981; Davies et al., 1980; Auclair et al., 1977a). Heterologous regulation of receptors may play an important role in the function of the rat corpus luteum particularly during pregnancy in which the endocrine gland is under the control of two hormones, LH and PRL (Rothchild, 1981; Morishige and Rothchild, 1974). The effects of these two luteotropic hormones are mediated through initial binding to their respective receptors. Apart from receptors for PRL and LH, receptors for both GnRH (Clayton et al., 1979) and $PGF_{2\alpha}$ (Wright et al., 1979) have also been identified in the rat corpus luteum. Although high levels of GnRH have been shown to cause up regulation of its receptor (Pieper et al., 1980), a decrease in both LH and PRL receptors after GnRH treatment has been observed in the immature rat primed with PMSG/hCG (Harwood et al., 1980d). A similar reduction in these receptors after $PGF_{2\alpha}$ treatment has also been reported (Behrman et al., 1971, 1978). The presence of several different receptors on the luteal cell surface suggests that the interaction between receptors may be of critical importance in our understanding of luteal function, especially during formation, desensitization and regression of the corpus luteum.

The physiological significance of down regulation may be to protect cells from exposure to high levels of circulating hormone

and so prevent excessive stimulation. Since both LH and PRL are known to maintain progesterone production in the rat corpus luteum it would be reasonable to assume that high levels (desensitization) of either hormone would cause a coordinated loss of both receptors from the luteal cell surface (Davies et al., 1980), a phenomenon suggestive of a close physical association between them. Desensitization of gonadal function in the rat may be a consequence of exogenous hCG/LH injected into the animal (Hsueh et al., 1976; Conti et al., 1977a, b; Rao et al., 1977) or of the endogenous preovulatory gonadotropin surge. In addition, GnRH may have a desensitizing role through the release of large quantities of pituitary LH (Cusan et al., 1979), although a luteolytic function similar to $PGF_{2\alpha}$-induced luteal regression has been ascribed. Under desensitizing conditions the loss of LH and PRL receptors is transitory with restoration of both receptors occurring within days of treatment while under luteolytic conditions the loss of receptors is irreversible.

The present experiments were designed to compare the effects of hCG, GnRH and $PGF_{2\alpha}$ on the pattern and reversibility of PRL and LH receptor loss in the corpus luteum of PMSG/hCG primed immature rats. In addition, it was of interest to quantitatively examine the effect of GnRH through changes in serum progesterone and receptor levels. Alterations in these parameters may reflect a role that is similar to the effect of hCG in terms of secondary desensitization of gonadal tissue through the release of large quantities of LH from

the pituitary. Alternatively, these changes may also ascribe a direct luteolytic action on the corpus luteum, analogous to the effect of $PGF_{2\alpha}$. Morphological studies involving light microscopy and electron microscopy were carried out to discern differences between desensitization involving a transient, reversible loss of function and luteolysis involving an irreversible loss of function.

2. Materials and Methods

2.1. Materials

Pregnant mare serum gonadotropin (PMSG; 1780-2570 IU/mg) and human chorionic gonadotropin (hCG; 2570-3861 IU/mg) were obtained from Sigma Chemical Company (St. Louis, Missouri) and prepared by dissolving in 0.1% BSA/PBS to yield a concentration of 250 and 125 IU/ml, respectively. The concentration of hCG required for desensitization ranged from 6.5-9.7 µg/0.2 ml injection. Gonadotropin-releasing hormone (des Gly^{10}, [D - Ala^{6}] ethylamide GnRH), obtained from Sigma, was prepared by dissolving 100µg in 10ml 0.1% BSA/PBS. A 5 mg/ml solution of $PGF_{2\alpha}$ (Lutalyse, dinoprost tromethamine) came from the Upjohn Company, Kalamazoo, Michigan.

For the radioreceptor assays, [^{125}I]-hCG was used to assess LH binding sites. Carrier free [^{125}I] NaI came from Amersham (Arlington Heights, Illinois) at a concentration of 100mCi/ml in 0.1 N NaOH. hCG (CR121; 13,000 IU/mg) for radioiodination was obtained from the National Pituitary Hormone Program, NIH (Baltimore, Maryland) and initially prepared as 10µl aliquots (1µg/µl in PBS) and

stored at -80°C in polypropylene microcentrifuge vials (Fisher). Lactoperoxidase (Grade B, Calbiochem, Las Jolla, California; 20 IU/mg) for radioiodination was dissolved in deionized water and stored in 50µl aliquots (1mg/ml) in microcentrifuge tubes at -80°C. The activity of the solution was checked prior to freezing by taking the ratio of the spectrophotometric optical density at 412nm and 280nm with a normal value around 0.75. An excess of unlabelled hCG (Sigma; 3360 IU/mg) was used to determine nonspecific binding and was prepared in 500µl aliquots (1mg/ml) and stored at -20°C. To assess lactogen binding sites, [^{125}I]-hGH was obtained from Hazelton Laboratories, Vienna, Virginia (total activity = 90µCi ; 45µCi/µg) and was aliquoted into 1ml fractions and stored at -80°C. Prior to each binding study a 1ml aliquot was thawed and diluted appropriately using 0.1% BSA/PBS. In addition, several PRL binding studies were performed to demonstrate $MgCl_2$ dissociation of PRL receptors. For these experiments hGH (N.P.H.P., Baltimore, Maryland) in 10µg/50ml aliquots was radioiodinated with [^{125}I] NaI. For the PRL binding studies, unlabelled oPRL (31 IU/mg), obtained from Sigma, was used to assess nonspecific binding and was prepared in 500µl aliquots (1mg/ml) and stored at -20°C.

For the steroid assays, labelled progesterone ([$_{1,2,6,7}$ - ^{3}H] progesterone; 104 Ci/mmol) and estrogen ($_{2,4,6,7}$ - ^{3}H estradiol-17β; 99 Ci/mmol) were obtained from Amersham. Unlabelled progesterone and 17β-estradiol came from Sigma while both the progesterone antibody (G.D.N. #337 anti-progesterone-11-BSA serum) and estrogen antibody (G.D.N. #224 anti-estradiol-6-BSA serum) were supplied by Dr. Gordon

Niswender, Colorado State University, Fort Collins, Colorado. The antibody preparations came as 1ml of lyophylized serum and were re-suspended in distilled water to make a 100µl of a 1:10 dilution. Antibodies were stored at -80°C.

All other chemicals are fine grade and are listed in Appendix A.

2.2. Animals and Treatment Schedule

Immature Holtzman female rats (~25 days old at time of arrival and weighing 50-75 grams) were obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts). Rats were housed at room temperature in a 14 hour light, 10 hour dark cycle and fed rat chow and water ad libitum.

All rats were injected with 0.2ml, 50IU PMSG at 0900 hours. PMSG has FSH-like activity and stimulates follicular growth and development. This was followed 56 hours later (at 1700 hours) by 0.2ml, 25IU hCG (designated day 0) to supplement the LH endogenous surge. hCG has LH-like activity and causes rupture of the mature follicle and release of the oocyte. Seven days after PMSG treatment rats were divided into three groups of 3-5 rats/group with each group given one of the following treatment regimens (0900 hours):

Group I--25IU (0.2ml, 6-9µg)hCG/rat

Group II--2µg (0.2ml) GnRH (des Gly^{10}, [D-Ala^{6}]ethylamide GnRH/rat

GroupIII--1 or 2mg (0.2 or 0.4ml)$PGF_{2\alpha}$(Lutalyse)/rat

The remainder of the rats were injected with 0.2ml, 0.1% BSA/PBS and served as a control group. All injections were given subcutaneously.

The protocol described above was designed for three experiments with the major difference lying in the time points at which the rats were sacrificed and the duration over which the experiments were carried out. In experiments I and II rats were sacrificed in groups of three by decapitation over a 25 hour and 5 day period, respectively, beginning with three control rats immediately after injection of 0.1% BSA/PBS. This was continued every 5 or 24 hour intervals thereafter for all treatment groups. In experiment III rats were sacrificed in groups of five (control) or four (other treatment groups) over an eight-day period beginning with five control rats immediately after vehicle injection. All other sacrifices were carried out every second day thereafter, with the exception of a six-hour time point after the initial sacrifice. The treatment schedule for all three experiments is shown in Figure 1.

An additional study was carried out to determine the optimum dose of $PGF_{2\alpha}$ (Lutalyse) necessary to cause a decrease in LH and PRL receptors. In PMSG/hCG primed female rats, 0.22, 0.66, and 2.0 mg of $PGF_{2\alpha}$ was injected subcutaneously seven days after PMSG treatment. The number of rats treated at each level was 12. A further 16 rats were injected with 0.1%, BSA/PBS and served as a control group. Rats were sacrificed in groups of four by decapitation over a two-day period beginning with four control rats

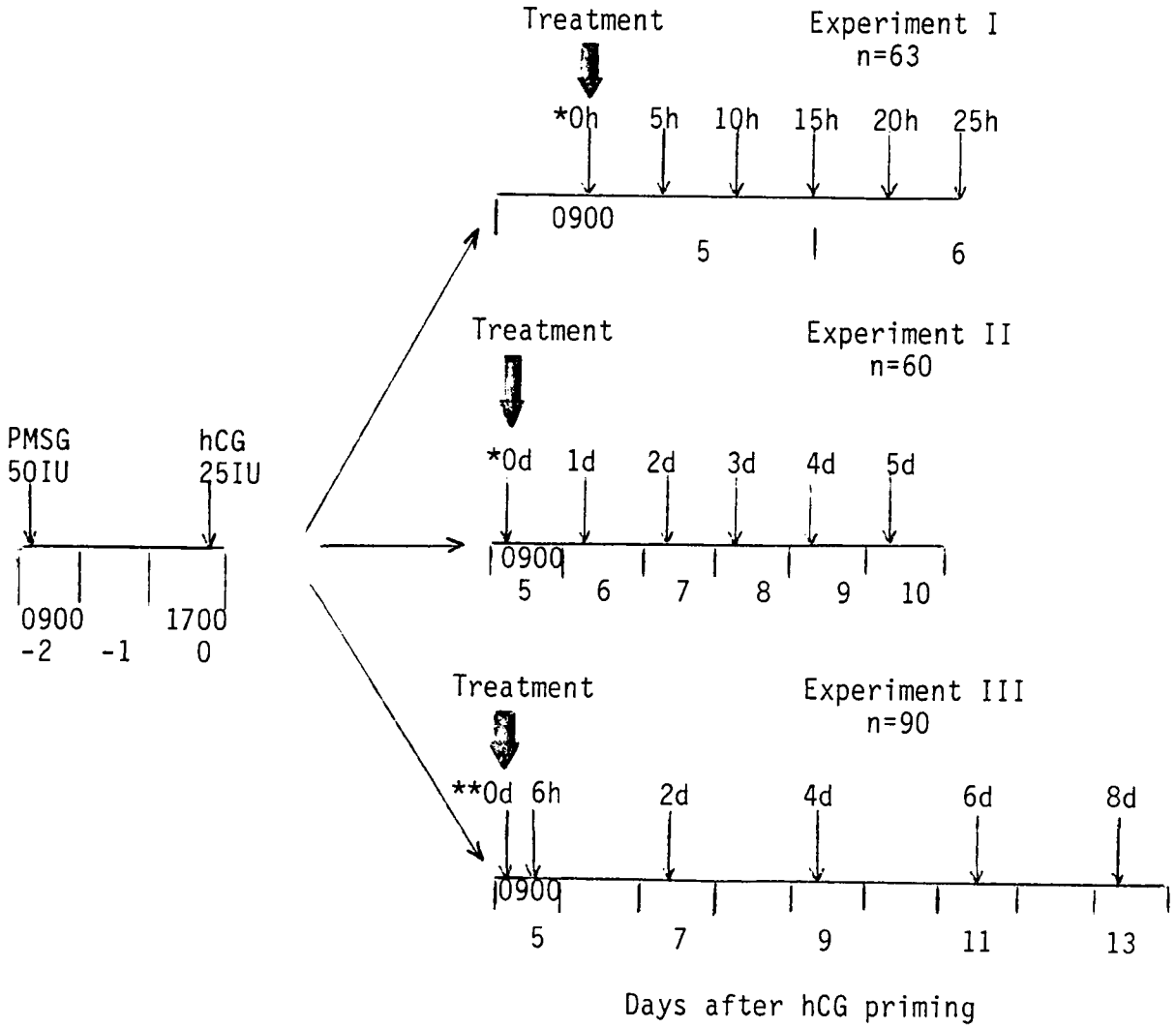

Figure 1. Schedule for the study of hCG, GnRH and $PGF_{2\alpha}$-treated superovulated rats, killed over a period of 25 hours. (0h-25h, Experiment I), 5 days (0d-5d, Experiment II) and 8 days (0d-8d, Experiment III). Day of hCG treatment = Day 0. At the time of treatment 3 (*) or 5 (**) control rats were sacrificed immediately.

immediately after injection of vehicle and continuing at six hours, one day and two days after treatment.

After sacrifice, the ovaries were removed, trimmed free of fat, connective tissue and oviduct. Both ovaries from rats in experiment III and one ovary from rats in experiments I and II were placed in a plastic vial and quick frozen on dry ice. Ovaries were stored at -80°C until assayed for LH and PRL receptors. The contralateral ovary from rats in experiments I and II was placed in a glass petri dish containing a small volume of 4% glutaraldehyde (GTA) in 0.1M sodium cacodylate buffer and sectioned into 6-8 pieces ($1mm^2$). Sections were then left in a clean scintillation vial containing 4% GTA for fixation and further processing for light and electron microscopy. Trunk blood, obtained from all rats and collected in 16 x 125mm (Fisher) test tubes was left at room temperature for 1-3 hours to prevent lysis of red blood cells and to allow formation of the clot. Blood samples were then refrigerated (4°C) and the following day serum was pipetted into 12 x 75mm borosilicate culture tubes (Fisher). Centrifugation (IEC HN-SII Centrifuge) of blood in original tubes was carried out at 1000 rpm for five minutes and additional serum obtained was pipetted into 12 x 75mm test tubes containing the bulk of the serum. Serum was frozen at -20°C until assayed for steroids.

2.3. Radioidination of hCG and hGH

Both hCG (CR121) and hGH were radioiodinated using the lacto-peroxidase method. Fifty microliters of 0.1M phosphate buffer

(pH 7.1) was added to a microcentrifuge tube containing hCG (10µg/10µl) or hGH (10µg/50µl) and mixed by finger tapping. Using a 10µl Hamilton syringe, 2mCi (20µl) [^{125}I] NaI was added to the surface of the hormone solution and mixed carefully. Hydrogen peroxide 40ng/10µl) and lactoperoxidase (10µg/10µl) were pipetted into the vial containing the unlabelled hormone and the reaction mixture allowed to incubate for five minutes at 40°C. After incubation 100µl transfer solution (16% sucrose) was added to the mixture to stop the reaction and the mixture then transferred to a Sephadex G-25 (5ml) column, previously equilibrated with 0.1% BSA/PBS. The vial containing the reaction mixture was washed with 200µl rinse solution (8% sucrose) and the remaining solution added to the top of the column. The column was drained (first fraction <1ml) and then 1ml 0.1% BSA/PBS was added to the column and drained again to obtain the second 1ml eluent. Elution of 1ml fractions was continued using 0.1% BSA/PBS, until 12 fractions were collected in 12 x 75mm test tubes. From each fraction 10µl was counted (with the pipette tip included) for radioactivity using a Beckman Gamma 4000 counter and the two highest readings in ascending order in the first peak (usually fractions 3 and 4) were either pooled, or separately aliquotted into 100µl samples and stored at -80°C. Aliquotting of high specific binding factions was necessary to avoid repetitive freezing and thawing of the radioiodinated hormone. Prior to each binding study an aliquot of labelled hormone was thawed and appropriate dilutions carried out

using 0.1% BSA/PBS. Radiolabelled hormones were used within three weeks of preparation.

2.4. Determination of LH/hCG Receptor Content by [^{125}I]-hCG Binding Assay

Radioiodinated hCG was used to assess LH binding sites since hCG and LH bind to the same receptors. [^{125}I]-hCG is more resistant to radioiodination damage and degradation than LH and has been shown to have higher affinity for rat gonadal LH receptors than either hLH or oLH (Huhtaniemi and Catt, 1981a).

Single ovaries, stored at -80°C from all rats were thawed (16 at a time) and weighed individually. Each ovary was homogenized in 4ml PBS (pH 7.4) using a Dounce homogenizer. The homogenate was transferred into a polycarbonate (16 x 98.7mm; Int. Equipment Company) tube and centrifuged at 27000 xg for 30 minutes at 4°C in a J-21C Beckman centrifuge. The supernatant was decanted and fatty material on the sides of the tubes removed. The crude membrane pellet was vortexed and resuspended in 2ml PBS.

Prior to carrying out the binding study 50µl (in triplicate) aliquots of membrane preparations were pipetted into 12 x 75mm test tubes for determination of protein content (Appendix B). In addition, several dilutions of membrane homogenate were prepared to determine the most appropriate concentration of membrane preparation necessary to give adequate binding (Appendix C).

One hundred microliters of each membrane homogenate was diluted 1:5 with PBS. Duplicate and triplicate tubes containing

100µl of the diluted homogenate were incubated (in 12 x 75mm test tubes) with 50µl of a saturating concentration of [^{125}I]-hCG (100,000cpm/50µl) with or without unlabelled hCG (10µg/10µl). Unlabelled hCG was used to measure nonspecific binding to glass. The binding for each homogenate was measured in triplicate for total binding and duplicate for nonspecific binding. Appropriate volumes of 0.1% BSA/PBS were added to all tubes so that the total reaction volume was 350µl. BSA/PBS was used as the buffer, since a previous study showed it reduced further the nonspecific binding when compared with PBS (Appendix D). In addition, the receptor assay was set up on ice to reduce the chance of degradation of the homogenate. The time between initial homogenization and the addition of hormones to all tubes was approximately 3-4 hours. The tubes were shaken and allowed to incubate overnight at room temperature. At the end of the incubation period (17 hours) the reaction mixture was diluted with 3ml cold 0.1% BSA/PBS and centrifuged (Beckman TJ-6R tabletop) at 3000 rpm for 30 minutes to separate unbound hormone in the supernatant from membrane bound hormone in the pellet. The supernatant was aspirated and the pellet counted for radioactivity in a γ-spectrometer (Beckman Gamma 4000; counting efficiency 70%). Specific binding was determined by subtracting nonspecific binding from total binding. Results are expressed as percent of control and in Appendix E as absolute values (cpm x 10^{-3}).

2.5 Determination of PRL Receptor Content by [^{125}I]-hGH Binding Assay

For the PRL receptor assays radiolabelled hGH was used because it is more stable than radiolabelled PRL. It has similar lactogenic activity to PRL and has been used to identify PRL receptors in a variety of target organs.

To determine PRL binding, ovarian homogenates were prepared as described earlier for the LH/hCG radioreceptor assay except that no dilutions were made. One hundred microliter samples (100µg protein) of crude membranes were incubated with 50µl [^{125}I]-hGH (100,000cpm/ 50µl) in the presence or absence of excess unlabelled oPRL (10µg/ 10µl). Buffer (0.1% BSA/PBS) in appropriate volume was added to all tubes for a final reaction volume of 350µl. For each homogenate three tubes were used to measure total binding and two tubes used to measure nonspecific binding. Tubes were shaken and incubated at room temperature for 17 hours. At the end of incubation, 3ml of 0.1% BSA/ PBS was added to all tubes and specific binding was calculated as previously described in the hCG binding study. The results are expressed as percent of control and in Appendix E as absolute values cpm x 10^{-3}).

The optimum time and temperature for the assay were determined in an earlier study by incubating ovarian membranes (100µg) with 75,000 cpm of [^{125}I]-hGH in the presence or absence of excess unlabelled oPRL at 5, 25 and 37°C. Incubation was carried out to various time points over 24 hours. Specific binding was determined

as previously described and the results expressed as a percentage of the total counts added.

In several initial PRL binding studies there was little binding of newly radioiodinated [^{125}I]-hGH. A possible explanation for these results was thought to be due to prior saturation of binding sites by endogenous PRL. With this in mind several studies were initiated in which 50µl of radiolabelled hGH (~100,000cpm), in the presence or absence of excess unlabelled oPRL (10µg/10µl) was added to tubes containing 50-200µl of membrane homogenate. The volume of the reaction was corrected for all tubes to 350µl with 0.1% BSA/PBS. After overnight incubation at room temperature, 3ml of 0.1% BSA/PBS was added to each tube. The suspension was centrifuged and the supernatant discarded as described earlier. Bound radioactivity was determined using a gamma counter. To dissociate receptors, 0.5ml of 4M $MgCl_2$ was added to the pellet and vortexed thoroughly. After five minutes, 3.5ml of 0.1% BSA/PBS was added to dilute the salt and the suspension was recentrifuged. The supernatant was aspirated and the pellet counted for radioactivity. A second wash in 0.1% BSA/PBS was carried out followed by further centrifugation and aspiration of the supernatant. The pellet was again counted for radioactivity prior to rebinding with 50µl of [^{125}I]-hGH with or without excess oPRL. Accordingly, the volume of the reaction mixture was adjusted to 350µl with 0.1% BSA/PBS. After a second overnight incubation the reaction was stopped with 3ml of 0.1% BSA/PBS and centrifugation

and aspiration of the supernatant carried out prior to determination of radioactivity in the pellet. Although the results of several of these experiments (Appendix F) suggest that the receptors may have been bound by endogenous PRL, alternative studies have shown no increase in binding after $MgCl_2$ treatment. Therefore, $MgCl_2$ was not used in subsequent binding studies.

2.6. Determination of Specific Activity of [^{125}I]-hGH and [^{125}I]-hCG

The specific activity of [^{125}I]-hGH and [^{125}I]-hCG were determined by progressively displacing radiolabelled hormone bound to rat luteal receptors with increasing concentrations of unlabelled oPRL and hCG, respectively. Unlabelled oPRL (0.2-500ng/100µl) or hCG (CR121) (0.32-20ng/100µl) were added to triplicate tubes containing 100µl of membrane preparation (diluted 1:5 for determining the specific activity of [^{125}I]-hCG), 100µl 0.1% BSA/PBS and 50µl of the respective labelled hormone (~100,000 cpm/50µl). Additional tubes, representing total binding, were set up as described above with the exception that no unlabelled hormones were added. Incubation was carried out at room temperature for 17 hours. The reaction was stopped with 3ml 0.1% BSA/PBS and unbound hormone separated from bound hormone by centrifugation (Beckman TJ-R tabletop) at 3000 rpm for 30 minutes. The supernatant containing unbound hormone was aspirated and the pellet counted for radioactivity as previously described in the radioreceptor assays for LH and PRL. An inhibition curve was generated by

designating the radioactivity bound to membranes in the absence of the competing unlabelled hormone as 100% binding (i.e., buffer control, % B_o/T) (Diekman et al., 1978a). Radioactivity was displaced proportionally with increasing concentrations of unlabelled hormone and expressed as a percentage of the buffer control. The amount of oPRL (or hCG) that displaced 50% of the labelled hGH (or hCG) from the receptor was determined.

In addition to the inhibition curve, increasing amounts of [^{125}I]-hGH and [^{125}I]-hCG, respectively were added to a constant membrane fraction (100µg) to saturate available receptors. Increasing concentrations of [^{125}I]-hGH and [^{125}I]-hCG in serial dilution (0.16×10^6-1.0×10^6cpm/100µl) were added to five tubes, two of which contained 10µg/10µl of unlabelled oPRL (or hCG) to monitor nonspecific binding. The final volume in all tubes was brought up to 350µl with 0.1% BSA/PBS. Tubes were shaken and incubated for 17 hours at room temperature. The amount of radioactivity bound to the membrane was determined as previously described. Specific binding at each concentration of radiolabelled hormone was determined by subtracting nonspecific binding from total binding and was expressed as a percentage of the total amount of radiolabelled hormone added to each tube (B_o/T). The B_o/T ratio obtained with the smallest amount of labelled hormone added was defined as 100% (Diekman et al., 1978a). As the concentration of [^{125}I]-hGH (or [^{125}I]-hCG) increased, the B_o/T ratio became smaller and each was expressed as a percentage of the B_o/T tubes that contained the smallest concentration of radiolabelled hormone.

The quantity of [^{125}I]-hGH (or [^{125}I]-hCG) added that gave a B_o/T ratio of 50% was determined.

The specific activity of these radiolabelled hormones was calculated by dividing the cpm obtained at a B_o/T ratio of 50% by the quantity of hCG that displaced 50% of the radiolabelled hormones in the inhibition curve.

2.7. Determination of Serum Levels of Progesterone

a. Extraction of progesterone. Serum samples (0.5ml) were extracted with 5ml of hexane in 13 x 100mm test tubes. A trace of ^{3}H-Progesterone (1000cpm/50µl) was added to monitor procedural losses. The tubes were vortexed and left overnight. Acetone and dry ice were used to freeze the lower aqueous phase from the upper organic phase containing progesterone. The non-aqueous phase was decanted into 12 x 75mm test tubes and dried under a gentle stream of air. The above procedure was repeated several times. The dried extract was reconstituted with 2ml PBS (pH 7.4) and the tubes vortexed and left overnight. One half milliliter of resuspended serum extract was placed in a 4ml polypropylene Wheaton Omnivial together with 0.5ml dioxane and 2.5ml toluene. The amount of radioactive progesterone recovered was determined by counting omnivials for 10 minutes using a liquid scintillation counter (Beckman LS-230). Control tubes containing 0.5ml of hexane in place of serum samples were included in the extraction. The recovery of ^{3}H-Progesterone

(and hence serum progesterone) was calculated as a percentage of the total counts added.

b. Progesterone assay.

Preparation of standard solutions: A 1µg/ml solution of unlabelled progesterone was prepared in absolute ethanol; 160µl of the solution was pipetted into a clean scintillation vial and air dried to remove the ethanol. To prepare a 16µg/ml stock solution of progesterone, 10ml of PBS (pH 7.4) was added to the scintillation vial. Various concentrations of progesterone standards (8ng-8pg/ml) were prepared by serial dilution of the stock solution with PBS.

A 1:250 dilution of progesterone antibody (G.D.N. #337) was made by adding 2.4ml 0.1% BSA/PBS to 100µl of a 1:10 dilution of stock antibody. It is desirable that the antibody dilution used in all assays be such as to allow for 50% binding of total radiolabelled progesterone added (50% buffer control). This is necessary since aliquots of progesterone antibody (100µl, 1:10 dilution) stored for long periods of time at -80°C may show considerable variation in buffer control values. Subsequently, prior to serum progesterone analysis, different dilutions of the antibody were prepared to determine the dilution required to give 50% bindability (see Appendix G).

Radiolabelled progesterone was prepared by air drying 10µl of ^{3}H-Progesterone in a clear glass scintillation vial and resuspending in 10ml of 0.1% BSA/PBS. After thorough mixing, 50µl of the tracer was placed in an Omnivial together with 0.5ml dioxane and 2.5ml toluene and radioactivity counted in a liquid scintillation

counter for one minute. Appropriate dilutions of the stock tracer solution were carried out using 0.1% BSA/PBS until approximnately 10,000cpm/50µl was obtained.

Progesterone assay procedure: Two hundred and fifty microliters of each standard progesterone concentration was added in triplicate to 12 x 75mm test tubes with the concentration ranging from 2ng to 2pg/tube. Additional tubes representing total and non-specific binding and buffer control were also included in establishing the standard curve. All standard curve tubes received 50µl of ^{3}H-Progesterone while 50µl of the 1:250 dilution of progesterone antibody was added to all except "total" and "nonspecific binding" tubes; 0.1% BSA/PBS was added to "total and nonspecific binding" and buffer control tubes so that the final reaction volume was 350µl.

In the corresponding assay of samples containing serum extract 250µl of sample together with 50µl of ^{3}H-Progesterone and 50µl of progesterone antibody was added in triplicate to 12 x 75mm test tubes so that the total volume of the reaction was 350µl.

Standard and sample test tubes were shaken vigorously and the reaction mixture was allowed to incubate for approximately 17 hours at 4°C. After incubation, 0.5ml of freshly prepared dextran coated charcoal solution was added to all tubes except "total counts" which received instead 0.5ml 0.1% BSA/PBS. The tubes were shaken and allowed to sit for 5-10 minutes. Unbound radioactive progesterone adsorbed to charcoal was removed from solution by centrifugation (Beckman TJ-6R Tabletop) at 3000 rpm for

30 minutes and the supernatant containing bound hormones decanted into polypropylene Omnivials. Dioxane (0.5ml) and toluene (2.5ml) were added to all vials which were then capped and vortexed thoroughly. The amount of radioactivity was determined in a liquid scintillation counter. The levels of progesterone were calculated by computer analysis (Faden and Rodbard, 1975) and corrected for the amount recovered during the extraction procedure. The results are expressed as percent control and in Appendix H as absolute values (ng/ml).

2.8. Determination of Serum Levels of Estrogen

Estrogen was extracted from serum with diethyl ether as the organic solvent using the same procedure described earlier for the extraction of progesterone. Fifty microliters of ^{3}H-estradiol (~1000cpm) was added to monitor the recovery of estrogen during the procedure.

In the analysis of serum estrogen, standard concentrations of 17 β-estradiol (8ng-8pg/ml) were prepared from a stock solution of 16ng/ml using the same procedure described for the preparation of standard solutions of progesterone. Estrogen antibody (G.D.N. #244; 100µl, 1:10 dilution) was diluted with 34.9ml 0.1% BSA/PBS to obtain a 1:3500 dilution. Tritiated estradiol was prepared by air drying 20µl of stock and resuspending in 20ml 0.1% BSP/PBS. The protocol for establishing and carrying out the assay for estrogen in both standard and sample tubes was identical to that used for progesterone.

2.9. Preparation of Tissue for Light and Electron Microscopy

Ovaries for light and electron microscopy were sectioned (~1-2mm^2) and placed in vials containing 4% glutaraldehyde (pH 7.4) in 0.1M sodium cacodylate buffer for two days at 4°C. Sections were then removed and washed twice in 0.1M sodium cacodylate buffer containing 5% sucrose for 10 minutes each with a further rinse in 0.1M sodium cacodylate buffer (without sucrose) for 10 minutes. Tissue was then post fixed with 1% OsO_4 solution in 0.1M sodium cacodylate buffer for two hours followed by two more 10 minute rinses in 0.1M sodium cacodylate buffer. The procedures up to this point were all carried out at 4°C.

Initial dehydration was carried out in 50% ethanol for 30 minutes at room temperature. Tissues were then stained for two hours in 2% uranyl acetate (prepared in 70% ethanol). Further dehydration was performed for 30 minutes each in 70, 80, 90, and 95% ethanol followed by final dehydration in 100% ethanol (3 changes, 30 minutes each). Tissues were then infiltrated (twice, 15 minutes each) in propylene oxide and allowed to sit overnight in a 1:1 mixture of propylene oxide and plastic (polybed). After further overnight infiltration in a 2:1 mixture of plastic and propylene oxide and pure plastic, the tissue was removed and placed in gelatin capsules (Size 00, 1:0cm^3, Polysciences) containing freshly prepared plastic. Capsules were placed in a vacuum overnight and later left in an oven for two days at 60°C.

For thick sectioning the gelatin capsule containing embedded tissue was removed and the tissue sectioned for light microscopy into approximately 0.5µm thick sections using a Sorvall UT-2 ultramicrotome. Sections were gently added to a drop of water on a slide and heated at 300°F to allow the tissue to stick to the slide. Staining was carried out on a hot plate using a mixture of 0.5% Azure blue and 1% borax. For electron microscopy 90nm thick sections were obtained using a Riechert OMU3 ultramicrotome. Sections were placed on copper mesh (400 and 300 mesh 3.05mm, Polysciences Inc.) grids for electron microscopy.

Light microscopic photographs (Panatomic X, ASA 32) of selected, stained thick sections were taken at 500 and 1250 X magnification using a 35mm camera attached to the Leitz Wetzler microscope. Prior to taking photographs using the electron microscope, thin sections on copper grids were further stained in uranyl acetate for 30' followed by several rinses in distilled water. Sections were then placed in a lead citrate solution for 5 minutes and then rinsed in distilled water and dried. Photographs of thin sections were taken using the Hitachi electron microscope at 60Kv.

2.10. Statistical Methods

To determine differences between treatments at each time point data were analyzed by paired student's t-test (Snedecor and Cochran, 1968).

3. Results

3.1 Determination of the Specific Activity of ^{125}I-hCG and ^{125}I-hGH

The specific activity of ^{125}I-hCG and ^{125}I-hGH was determined by radioreceptor assay using the procedure described by Diekman et al., 1978a). In determining the specific activity of the radioactive hormones, 2ng of unlabelled hCG (Figure 2, curve A) and 6.2ng of unlabelled oPRL (Figure 3, curve A) displaced 50% of ^{125}I-hCG and ^{125}I-hGH, respectively, from luteal membranes. The amount of radioactivity bound to excess membranes in the absence of competing unlabelled hormone is defined as 100% (Diekman et al., 1978a). In addition, as increasing concentrations of ^{125}I-hCG and ^{125}I-hGH were added to membranes, there was a progressive decrease in the B_o/T ratio. (B_o/T is defined in the Materials and Methods and includes the smallest amount of labelled hormone added to membranes.) A B_o/T of 50% was obtained with 950,000cpm ^{125}I-hCG (Figure 2, curve B) and 140,000cpm ^{125}I-hGH (Figure 3, curve B), respectively. The specific activity of ^{125}I-hCG and ^{125}I-hGH was determined to be 309μCi/μg and 14.7μCi/μg, respectively. Each value was calculated by dividing the counts necessary to cause a B_o/T of 50% by the amount of unlabelled hormone needed to displace 50% of the radioactive hormone from luteal membranes (Figures 2 and 3).

Figure 2. Determination of the specific activity of ^{125}I-hCG. Curve A represents an inhibition curve generated by adding increasing amounts of unlabelled hCG (0.32-20ng) to tubes containing 100,000cpm of ^{125}I-hCG (~1ng) and membrane homogenate. Curve B illustrates the addition of increasing concentrations of ^{125}I-hCG (0.16×10^6-1×10^6 cpm to tubes containing membranes with or without an excess of unlabelled hCG. The specific activity of ^{125}I-hCG was calculated by dividing the cpm obtained at a B_o/T ratio of 50% by the quantity of hCG that displaced 50% of the radiolabelled hormone.

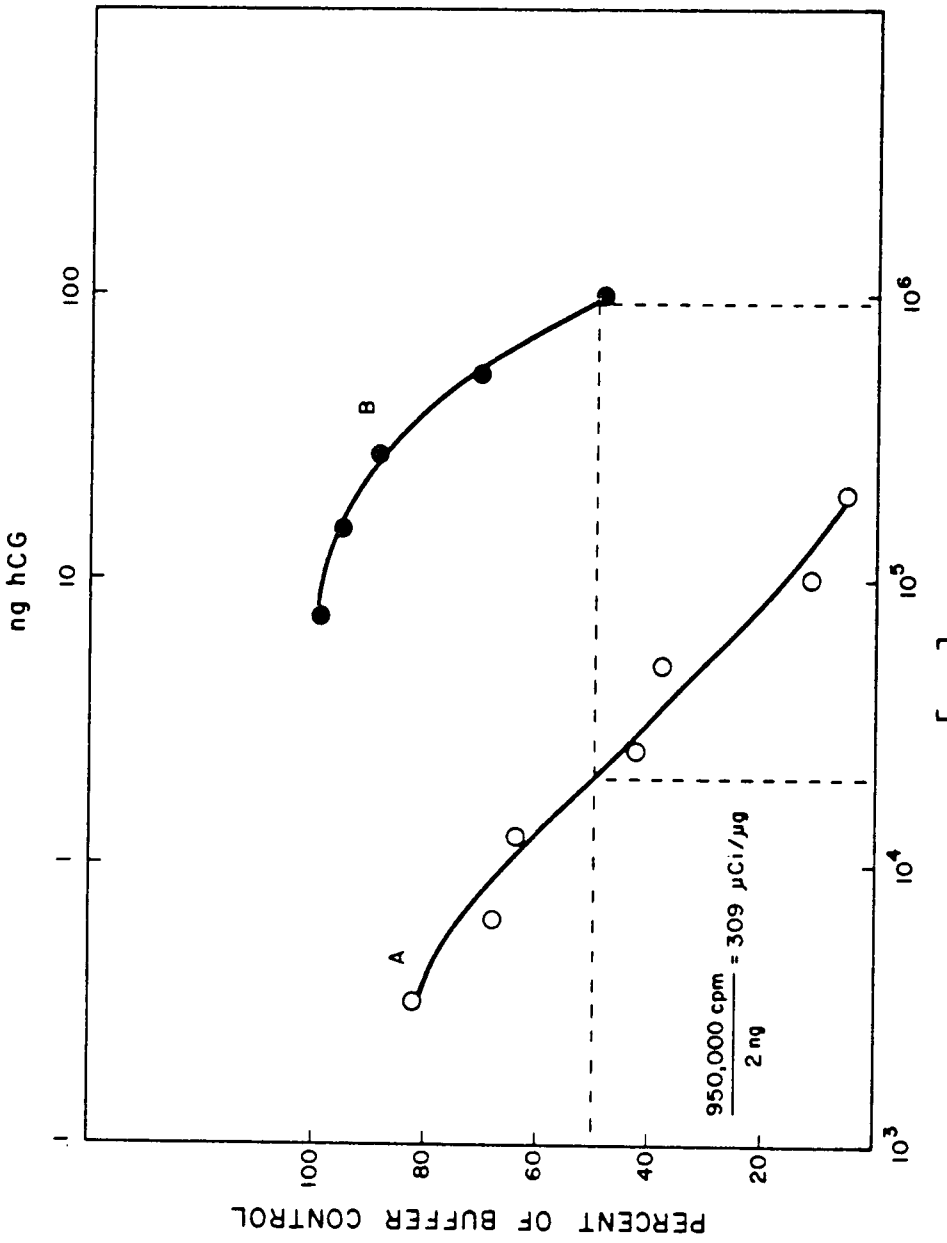
ng hCG
PERCENT OF BUFFER CONTROL
[125I]-hCG (cpm)
A
B
950,000 cpm / 2 ng = 309 µCi/µg

Figure 2

Figure 3. Determination of the specific activity of ^{125}I-hGH. An inhibition curve (Curve A) was generated by adding increasing amounts of unlabelled oPRL (.2ng-500ng) to tubes containing approximately 1ng of ^{125}I-hGH and membrane homogenate. Curve B was obtained by adding increasing concentrations of ^{125}I-hGH (0.16×10^6-1×10^6cpm) to tubes containing membranes in the absence or presence of unlabelled oPRL (10µg). The specific activity of ^{125}I-hGH was determined as described in the legend for Figure 2.

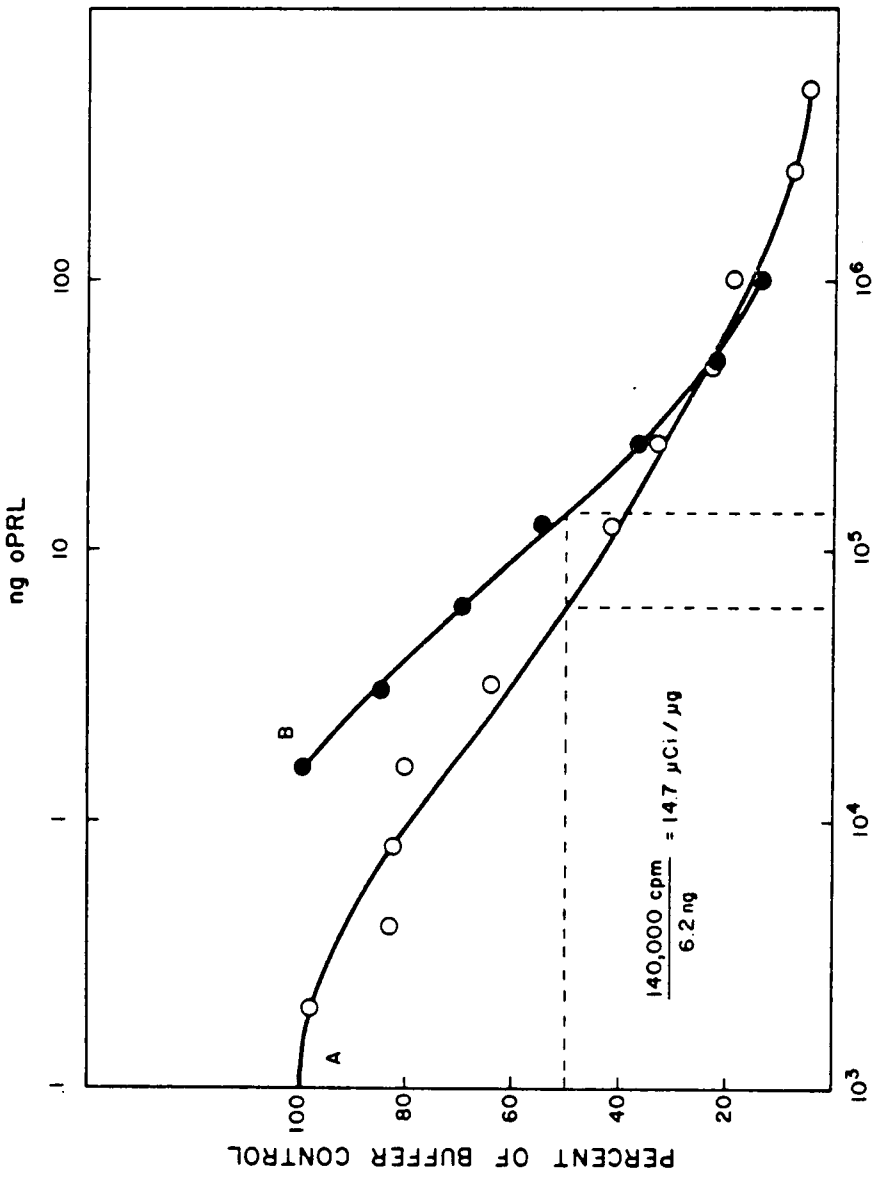
PERCENT OF BUFFER CONTROL
100
80
60
40
20
ng oPRL
1
10
100
A
B
$\frac{140,000 \text{ cpm}}{6.2 \text{ ng}}$ = 14.7 µCi / µg
10^3
10^4
10^5
10^6
[^{125}I]-hGH (cpm)

Figure 3

3.2. Kinetics of hGH Binding to Receptor Sites in Rat Luteal Tissue

The optimum time and temperature for ^{125}I-hGH binding to rat luteal homogenates is shown in Figure 4. Maximum binding of ^{125}I-hGH was obtained at 25°C with equilibrium occurring at approximately 17-20 hours. Increasing the temperature to 37°C did not increase specific binding above that seen at 25°C. After 12 hours incubation at 37°C, specific binding gradually declined. Rose et al. (1983) have also reported similar results in mink uterine membranes using ^{125}I-oPRL as the binding ligand. At 5°C little binding of ^{125}I-hGH was observed. When expressed as a percentage of the total hormone bound over all the times studied, nonspecific binding was greatest at 37°C (38 ± 16%). At 5°C and 25°C nonspecific binding was 32 ± 14.8% and 17 ± 6.2%, respectively. Since maximum specific binding occurred at 25°C all future PRL binding studies were performed at 25°C for 17 hours.

3.3. The Effect of Different Doses of $PGF_{2\alpha}$ on LH and PRL Receptors in the Corpus Luteum of Superovulated Rats

A dose response study was carried out to determine the optimum level of $PGF_{2\alpha}$ required to cause a prolonged reduction in binding sites for PRL and LH. The effect of different doses of $PGF_{2\alpha}$ on the number of LH receptors over a period of 48 hours is shown in Figure 5 (graph A) and in Table E-1, Appendix E. Rats treated with either 0.22, 0.66, or 2.0mg of $PGF_{2\alpha}$ exhibited a significant decrease (10-15% of control; $P<0.1$) in binding of ^{125}I-hCG within six hours of treatment. At 0.22 and 0.66 mg doses, binding

Figure 4. The effect of time and temperature on binding of ^{125}I-hGH to rat luteal membranes. A constant amount (100µg) of membrane protein was incubated with 75,000cpm of ^{125}I-hGH in the presence or absence of 10µg of unlabelled oPRL at 5, 25, and 37°C over 17 hours. Specific binding was determined by subtracting the amount of radioactivity bound to membranes incubated in the presence of excess unlabelled hormone (nonspecific binding) from the amount of radioactivity bound to membranes in the absence of unlabelled hormone (total binding). Results are expressed as percent of total counts added and each point represents the mean of three replicates.

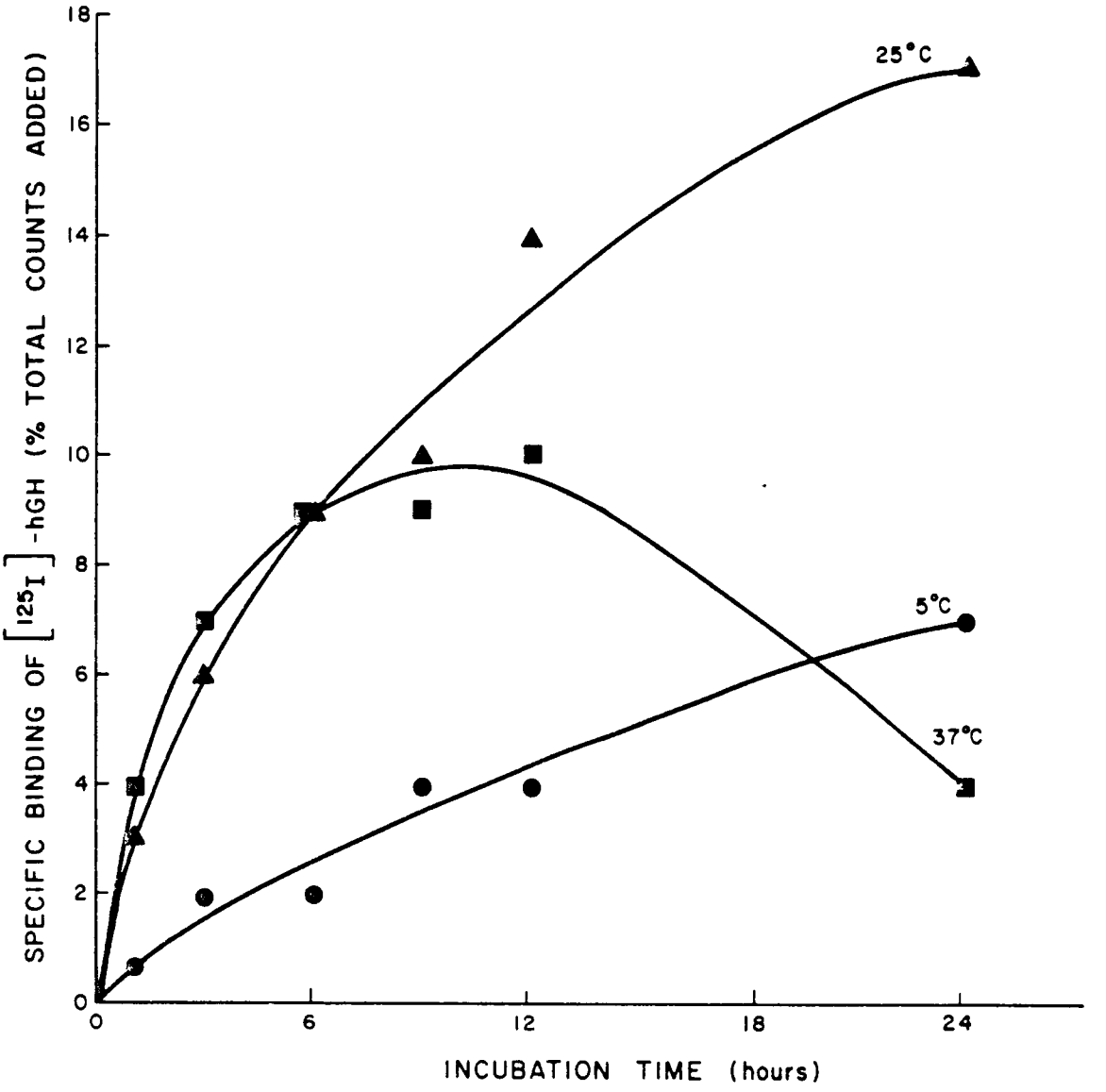
SPECIFIC BINDING OF [125I]-hGH (% TOTAL COUNTS ADDED)
INCUBATION TIME (hours)
25°C
5°C
37°C
0
2
4
6
8
10
12
14
16
18
0
6
12
18
24

Figure 4

of ^{125}I-hCG returned to control values between 24 and 48 hours. At the highest dose of 2.0mg of $PGF_{2\alpha}$, LH receptors were significantly reduced ($P<0.02$) by 20% at 24 hours compared to control levels, and exhibited a much slower return to control values compared to either of the two lower doses. A comparable pattern of loss and recovery for PRL binding sites is shown in Figure 5 (graph B) and in Table E-1, Appendix E. The decrease in ^{125}I-hGH binding at six hours was markedly greater (40-80% of control; $P<0.001$) compared to the effect of the three doses of $PGF_{2\alpha}$ on ^{125}I-hCG binding. In rats treated with 0.22 and 0.66mg of $PGF_{2\alpha}$, PRL receptors returned to control levels by 24 hours. Although a large decrease in binding sites for PRL was still evident 24 hours after treatment with 2.0mg of $PGF_{2\alpha}$, a more rapid rate of recovery was apparent compared to the recovery of binding sites for LH. Since the highest dose of $PGF_{2\alpha}$ used in this study produced the most pronounced effect in terms of a decrease in binding sites, all future experiments were carried out using either 1 or 2mg doses of $PGF_{2\alpha}$.

3.4. Effect of hCG, GnRH AND $PGF_{2\alpha}$ Treatment on LH Binding Sites

Prior to carrying out radioreceptor studies, the effect of hCG, GnRH and $PGF_{2\alpha}$ on the weight of individual ovaries was determined. In all experiments, there was no significant change in ovarian weight over different sampling times for each treatment or between different treatments at each time point. For these reasons, data on the effect of hCG, GnRH and $PGF_{2\alpha}$ on ovarian weight are not presented.

Figure 5. The effect of different doses of $PGF_{2\alpha}$ on the number of LH (graph A) and PRL (graph B) binding sites in the corpus luteum of superovulated rats. Membrane homogenate (20 or 100µg protein) was incubated with 1ng ^{125}I-hCG (or ^{125}I-hGH) with or without an excess of unlabelled hCG (or oPRL). Specific binding was determined as described in the legend for Figure 4. The results are expressed as percent of control. Each point represents the mean from four rats.

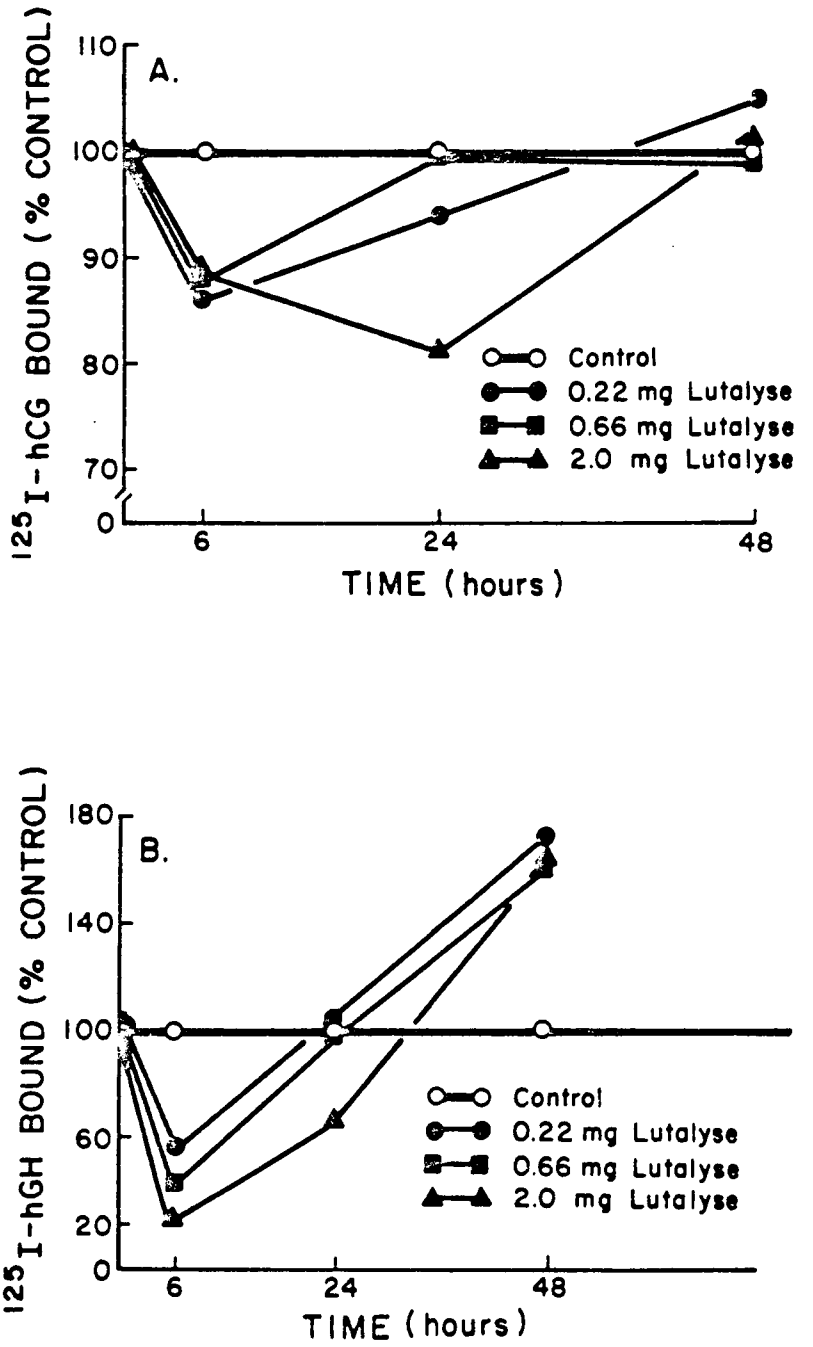
A.
110
100
90
80
70
0
^{125}I-hCG BOUND (% CONTROL)
6
24
48
TIME (hours)
Control
0.22 mg Lutalyse
0.66 mg Lutalyse
2.0 mg Lutalyse
B.
180
140
100
60
20
0
^{125}I-hGH BOUND (% CONTROL)
6
24
48
TIME (hours)
Control
0.22 mg Lutalyse
0.66 mg Lutalyse
2.0 mg Lutalyse

Figure 5

The effects of hCG, GnRH and $PGF_{2\alpha}$ treatment on the number of LH receptors were examined. In experiment I, binding of ^{125}I-hCG decreased gradually over the 25 hour period for all treatment groups (Figure 6 and Table E-2, Appendix E). In particular, a significant decrease ($P<0.05$) occurred between 15 and 25 hours after treatment. These results correlate well with the significant decrease in binding of ^{125}I-hCG ($P<0.02$) observed at day 1 in experiment II (Figure 7, graph A). hCG caused the greatest reduction in LH binding sites by day 2 (20% of control; $P<0.001$) compared to both GnRH and $PGF_{2\alpha}$ treatment. However, the decrease in receptors was transient since ^{125}I-hCG binding returned to control values within days 3 to 4. The luteolytic effect of $PGF_{2\alpha}$ was not fully expressed in this experiment since LH receptors returned to control levels between days 2 and 3. This result may reflect the low dosage of $PGF_{2\alpha}$ used in the experiment. Binding of ^{125}I-hCG was greatest (40-80% above control) at day 4 for all treatment groups. This result does reflect a true increase in receptor numbers after treatment, since values began to increase from day 2 onward after the initial decrease. However, these high values are misleading due to the large drop in the control value at day 4 (Table E-3, Appendix E) and are not significantly different when compared to the control group. By day 5 LH receptors decreased markedly in the GnRH treated group ($P<0.02$), while in rats treated with hCG and $PGF_{2\alpha}$ the change in LH receptors was not significant when compared to control values.

Figure 6. Experiment I: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on ^{125}I-hCG binding in the corpus luteum of the super-ovulated rat. Tissues were collected at 5 hourly intervals for 25 hours. The number of rats per treatment group at each time point is 3. The same procedure for the hCG binding study was carried out as described in the legend for Figure 5. Results are expressed as percent of control.

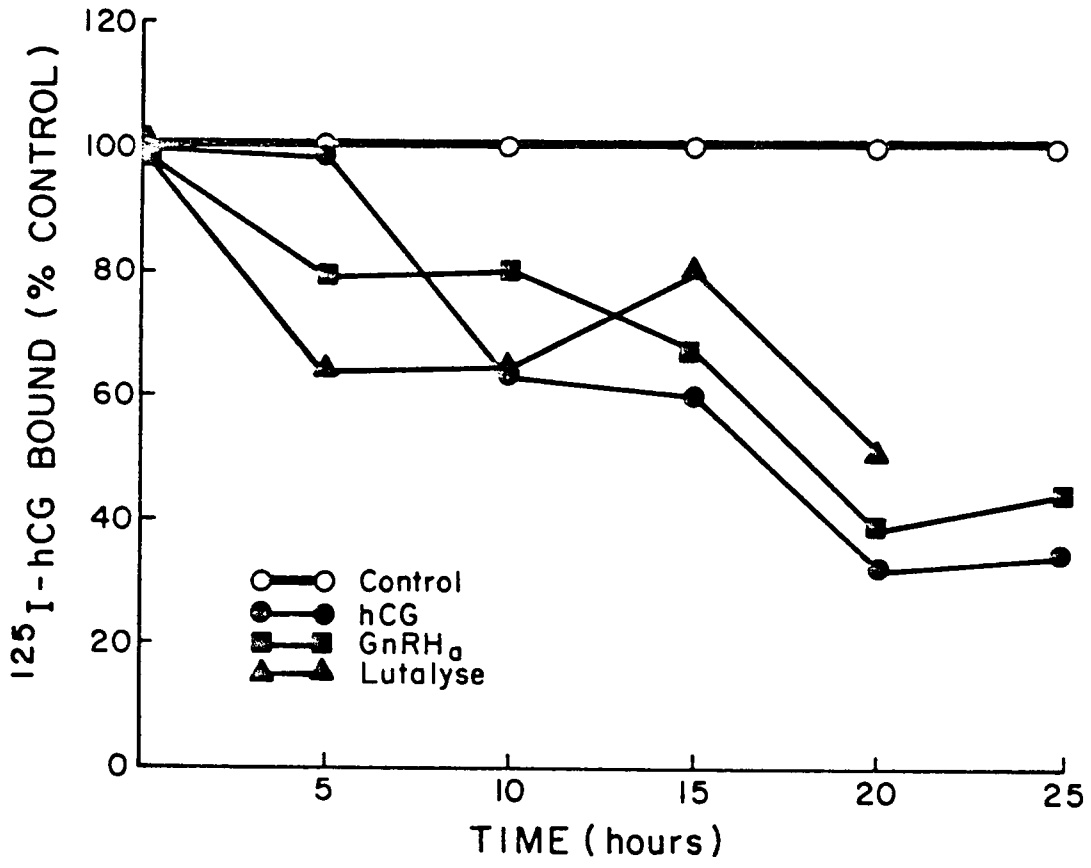
120
100
80
60
40
20
0
^{125}I-hCG BOUND (% CONTROL)
Control
hCG
GnRH$_a$
Lutalyse
5
10
15
20
25
TIME (hours)

Figure 6

Figure 7. Experiment II: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on the binding of ^{125}I-hCG (graph A) and ^{125}I-hGH (graph B) in the corpus luteum of the superovulated rat. Tissues were collected at 24 hour intervals for 5 days. The number of rats per treatment group at each time point is 3. The same procedure was carried out as described in the legend for Figure 5. Results are expressed as percent of control.

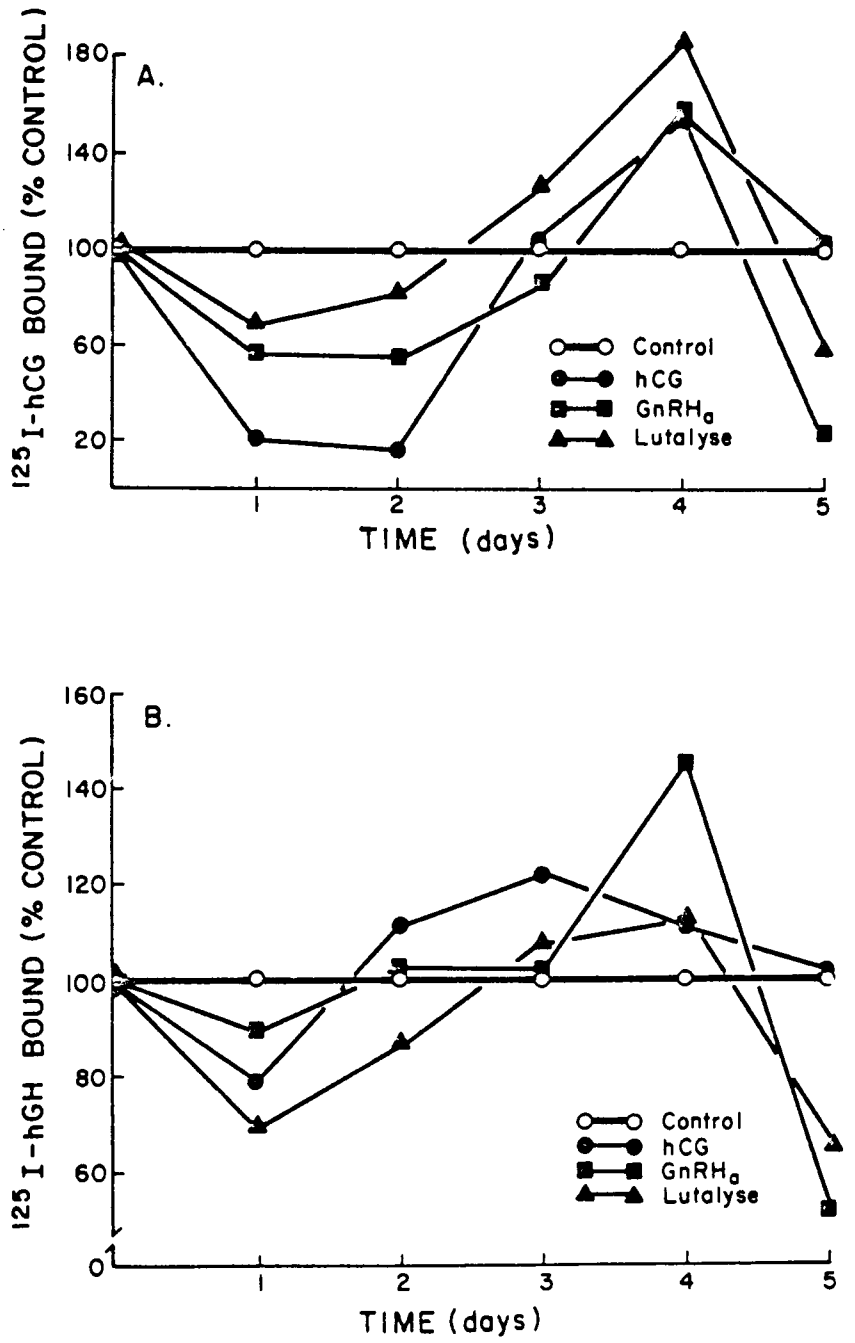
A.
^{125}I-hCG BOUND (% CONTROL)
180
140
100
60
20
1
2
3
4
5
TIME (days)
Control
hCG
GnRH$_a$
Lutalyse
B.
^{125}I-hGH BOUND (% CONTROL)
160
140
120
100
80
60
0
1
2
3
4
5
TIME (days)
Control
hCG
GnRH$_a$
Lutalyse

Figure 7

In experiment III, in which tissue was sampled over a period of 8 days, a corresponding initial reduction in ^{125}I-hCG binding sites occurred by day 2 in all treatment groups (Figure 8, graph A). As in experiment II, the greatest decrease in LH receptors occurred in rats treated with hCG ($P<0.001$). Recovery of receptors to control levels by day 4 was again evident in both hCG and GnRH treated rats. However, unlike the results obtained in experiment II, $PGF_{2\alpha}$ caused an irreversible decrease in LH receptors over the entire 8 day period, although a significant decrease was not observed until day 6 ($P<0.1$). The sustained reduction in LH receptors after $PGF_{2\alpha}$ treatment may be the result of the higher dosage used in this experiment compared to experiment II. From day 6 onwards LH receptors declined more rapidly in GnRH treated rats compared to hCG treated rats with a significant decrease ($P<0.05$) occurring only at day 8. This result corresponds to the difference in the rates of receptor decrease shown at the end of the time course in experiment II. The results from experiment III reflect a more accurate interpretation of treatment effect compared to experiment II since binding sites for ^{125}I-hCG in control rats remained relatively stable over the length of the study period (Table E-4, Appendix E).

3.5. The Effect of hCG, GnRH and $PGF_{2\alpha}$ on PRL Binding Sites

The initial decrease in PRL receptors followed by recovery parallels closely the pattern observed for LH receptors after treatment with $PGF_{2\alpha}$, GnRH and hCG. In experiment II, $PGF_{2\alpha}$

Figure 8. Experiment III: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on the binding of ^{125}I-hCG (graph A) and ^{125}I-hGH (graph B) in the corpus luteum of the superovulated rat. Tissues were collected at 6 hours after treatment and every second day thereafter following treatment for a period of 8 days. The number of rats per treatment group at each time point is 5 (controls) and 4 (other treatment groups). The same procedure was carried out as described in the legend for Figure 5. Results are expressed as percent of control.

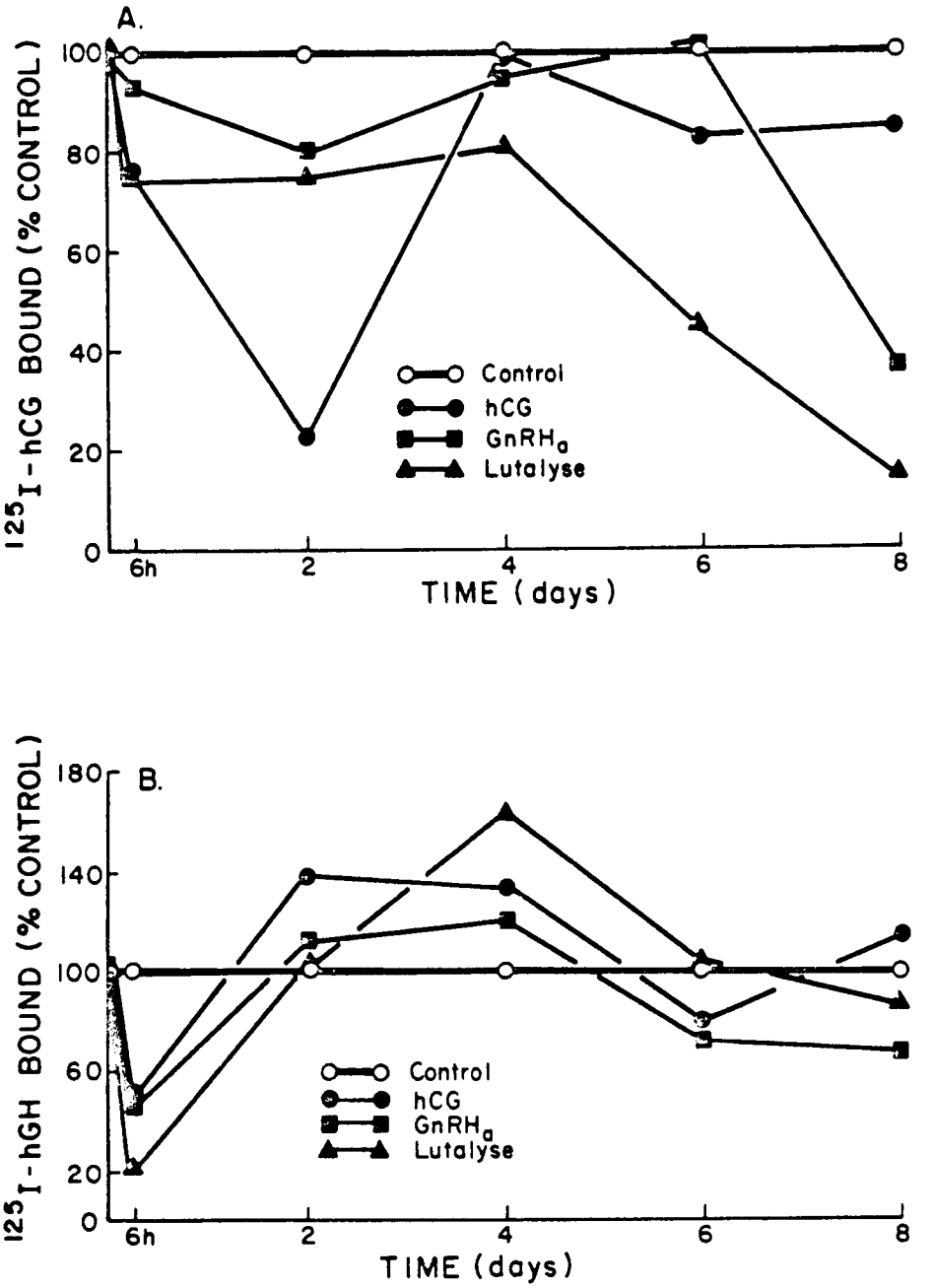
A.
^{125}I-hCG BOUND (% CONTROL)
100
80
60
40
20
0
6h
2
4
6
8
TIME (days)
Control
hCG
GnRH$_a$
Lutalyse
B.
^{125}I-hGH BOUND (% CONTROL)
180
140
100
60
20
0
6h
2
4
6
8
TIME (days)
Control
hCG
GnRH$_a$
Lutalyse

Figure 8

caused a significant reduction (50% of control; $P<0.05$) in PRL receptors by day 1 (Figure 7, graph B). The effect of hCG and GnRH in reducing ^{125}I-hGH binding at day 1 was not significant. This time point may reflect recovery of receptors since in experiment III a more marked decrease is evident 6 hours after treatment (Figure 8, graph B). In hCG and GnRH treated rats (experiment II) PRL receptors returned to control values by day 2. Recovery of PRL receptors after $PGF_{2\alpha}$ treatment did not occur until day 3. Binding of ^{125}I-hGH was highest at either day 3 in rats treated with hCG or at day 4 in the $PGF_{2\alpha}$ and GnRH treated groups. Although rats treated with GnRH showed a significant increase in PRL receptors above control levels at day 4 (40% above control; $P<0.1$), this result may not reflect altogether a treatment effect since control values for PRL receptors were reduced at this time (Table E-3, Appendix E). The reduction in PRL receptors exhibited by the control group from day 4 was similar to the decrease in LH receptors observed at this time. By day 5 a significant reduction in PRL receptors ($P<0.05$) below control levels was evident only in the GnRH treated group.

When tissue was sampled over 8 days, the changes in PRL receptors closely paralleled the change demonstrated in experiment II. A significant decrease in PRL receptors ($P<0.05$) at 6 hours for all treatment groups was followed by recovery to control values by day 2 (Figure 8, graph B; Table E-4, Appendix E).

Compared to the irreversible decrease in LH receptors observed over the 8 day sampling period, the loss of PRL receptors was transient. Although binding of ^{125}I-hGH was greatest at day 4 for all treatment groups, the increases were not significant ($P>0.1$) when compared to the control group. A gradual decline in PRL receptors occurred from day 4 to day 8. However, no significant difference between control and treated groups was apparent, reflecting a luteolytic rather than treatment effect.

3.6 Serum Progesterone and Estrogen Levels in Rats Treated with hCG, GnRH and $PGF_{2\alpha}$

Estrogen levels were initially determined to ensure that rats had been suitably primed with PMSG and hCG. Low levels of estrogen compared to progesterone would indicate that the bulk of ovarian tissue is luteal rather than follicular. In the three experiments investigated, the amount of estrogen present in the serum was negligible compared to the concentration of progesterone, suggesting that the ovary was largely luteal in nature.

Since many corpora lutea are present in the ovaries of rats treated with PMSG and hCG, changes in the levels of serum progesterone are an indication of the effect of hCG, GnRH and $PGF_{2\alpha}$ on luteal tissue.

In experiment I, GnRH and $PGF_{2\alpha}$ caused a significant decrease in serum progesterone levels (<50% of control; $P<0.02$) within 10 hours of treatment (Figure 9; Table H-1, Appendix H). In $PGF_{2\alpha}$ treated rats progesterone levels remained well below control values

Figure 9. Experiment I: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on serum progesterone levels in the superovulated rat. Blood was collected at 5 hourly intervals following treatment and continued for a period of 25 hours. Progesterone concentrations in extracted serum were determined by radioimmunoassay. The results are expressed as percent of control. Each point represents the mean of three rats.

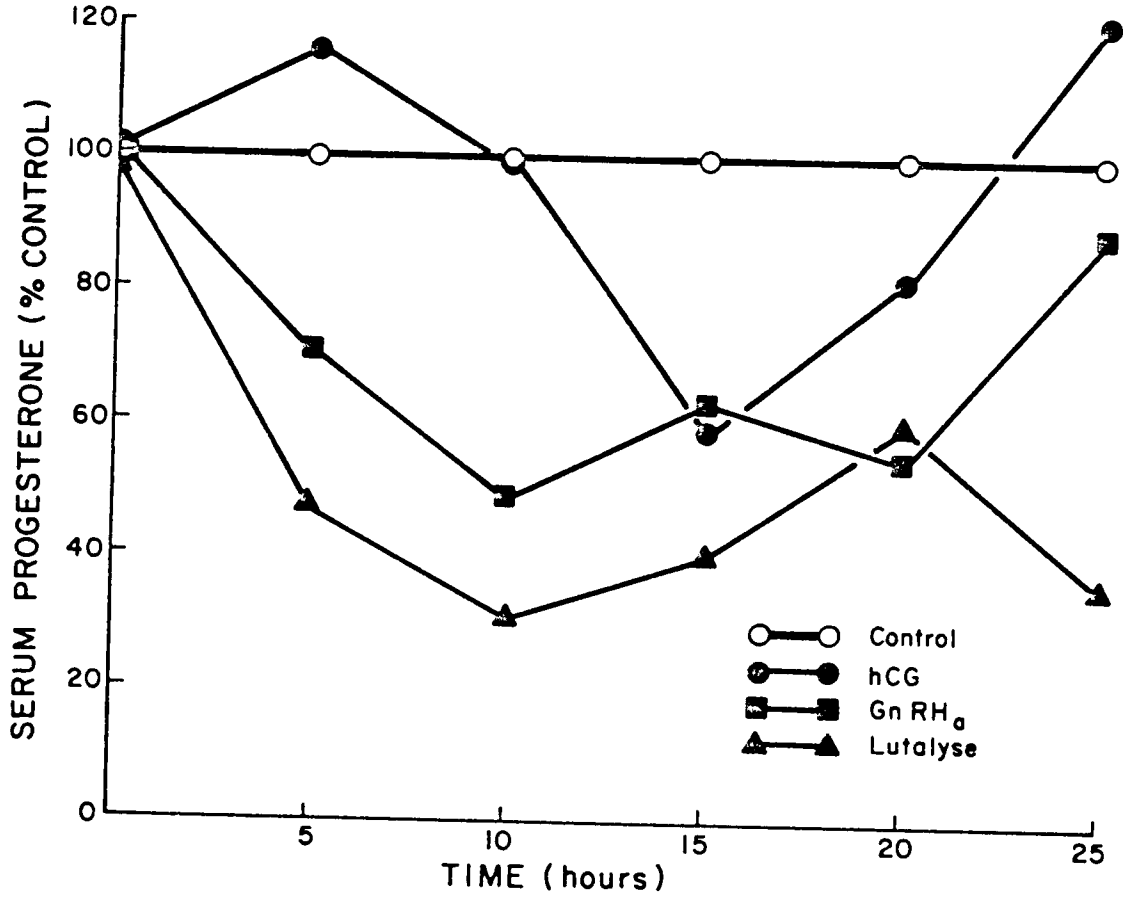
SERUM PROGESTERONE (% CONTROL)
120
100
80
60
40
20
0
5
10
15
20
25
TIME (hours)
Control
hCG
Gn RH$_a$
Lutalyse

Figure 9

over the entire 25 hour period. However, although rats treated with GnRH maintained progesterone levels below control values over the next 15 hours there were no significant differences when compared to control values. On the contrary, rats treated with hCG exhibited an initial increase in progesterone (20% above control) within 5 hours of treatment. This increase, however, was not significantly different from control values at this time. The rise in progesterone was followed by a marked decrease relative to control values over the next 10 hours ($P<0.1$ at 15 hours). At the end of the 25 hour period progesterone levels were once more above control.

In experiment II (Figure 10; Table H-2, Appendix H) a significant decrease in serum progesterone (<20% of control; $P<0.01$) was observed at day 1 after $PGF_{2\alpha}$ treatment. This result correlates well with the low levels observed at 25 hours in experiment I. However, over the next 3 days, serum levels of progesterone were not different from controls, although by day 5 progesterone levels were once again significantly reduced ($P<0.01$). In both hCG and GnRH treated rats an initial rise in progesterone over 1 and 2 days, respectively was observed. The rise in progesterone concentrations at day 1 in hCG treated rats was not significantly different when compared to control values. However, this initial change does correlate well with the rise in progesterone at 25 hours shown in experiment I. From day 1, however, serum progesterone decreased and remained at approximately 70% of control values ($P>0.1$) for the remainder of the study. A similar pattern was observed in rats

Figure 10. Experiment II: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on serum progesterone levels in the superovulated rat. Blood was collected at 24 hour intervals following treatment and continued for a period of 5 days. Progesterone concentrations in serum extract were determined by radioimmunoassay. Results are expressed as percent of control. Each point represents the mean of three rats.

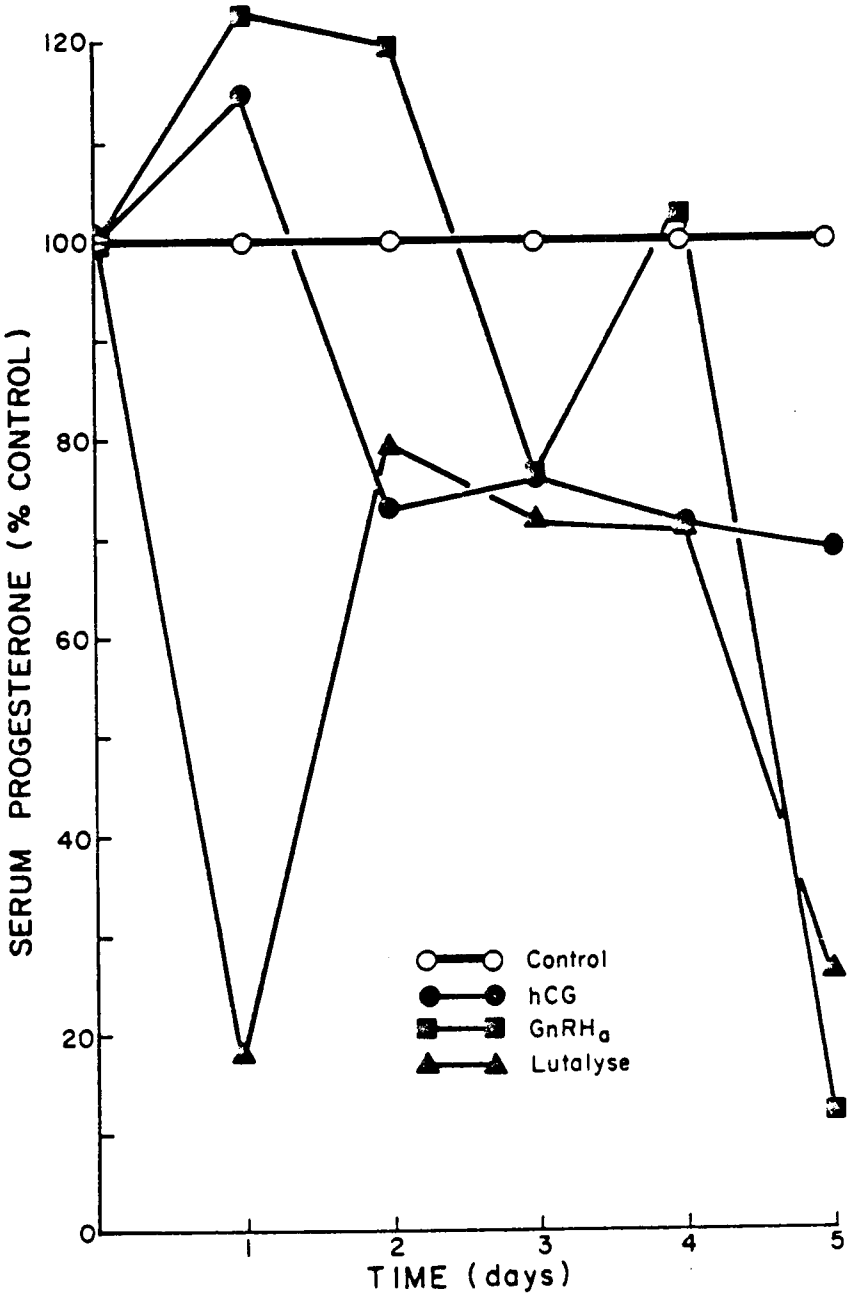
120
100
80
60
40
20
0
SERUM PROGESTERONE (% CONTROL)
1
2
3
4
5
TIME (days)
Control
hCG
GnRHa
Lutalyse

Figure 10

treated with GnRH. In these rats the initial rise in progesterone over the first 2 days ($P<0.1$ at day 1) was followed by a steady decline although a small but insignificant ($P>0.1$) increase was observed on day 4. By day 5 the level of progesterone in GnRH treated rats was significantly reduced ($P<0.001$).

In experiment III (Figure 11; Table H-3, Appendix H) the pattern of change in progesterone concentrations in rats treated with hCG, GnRH and $PGF_{2\alpha}$ corresponded closely with the results obtained from experiment II. The major difference lay in the initial changes in progesterone levels in rats treated with GnRH. At 6 hours after GnRH treatment a 20% decrease below control values was observed ($P<0.05$). This initial reduction in serum progesterone was not detected in experiment II as sampling did not begin until day 1 and the decrease may well have occurred before this time point. A marked stimulatory effect ($P<0.05$) can be seen at day 2 in experiment III in contrast to the smaller increase observed in experiment II. In GnRH treated rats progesterone levels continued to decline and by day 8 were significantly reduced ($P<0.1$). Rats treated with $PFG_{2\alpha}$ exhibited a marked decrease in progesterone levels which was significant ($P<0.1$) at all time points with the exception of days 4 and 6. In hCG treated rats a small but significant increase in progesterone ($P<0.1$) occurred within 6 hours of treatment. Although progesterone levels remained below control values for the remainder of the sampling period there was no significant difference between treatment and control groups.

Figure 11. Experiment III: the effect of hCG, GnRH, and $PGF_{2\alpha}$ on serum progesterone levels in superovulated rats. Blood was collected every 48 hours after treatment for a period of 8 days with an initial collection at 6 hours. Progesterone concentrations were determined by radioimmunoassay. Results are expressed as percent of control. Each point represents the mean of five (control) or four (other treatments) rats.

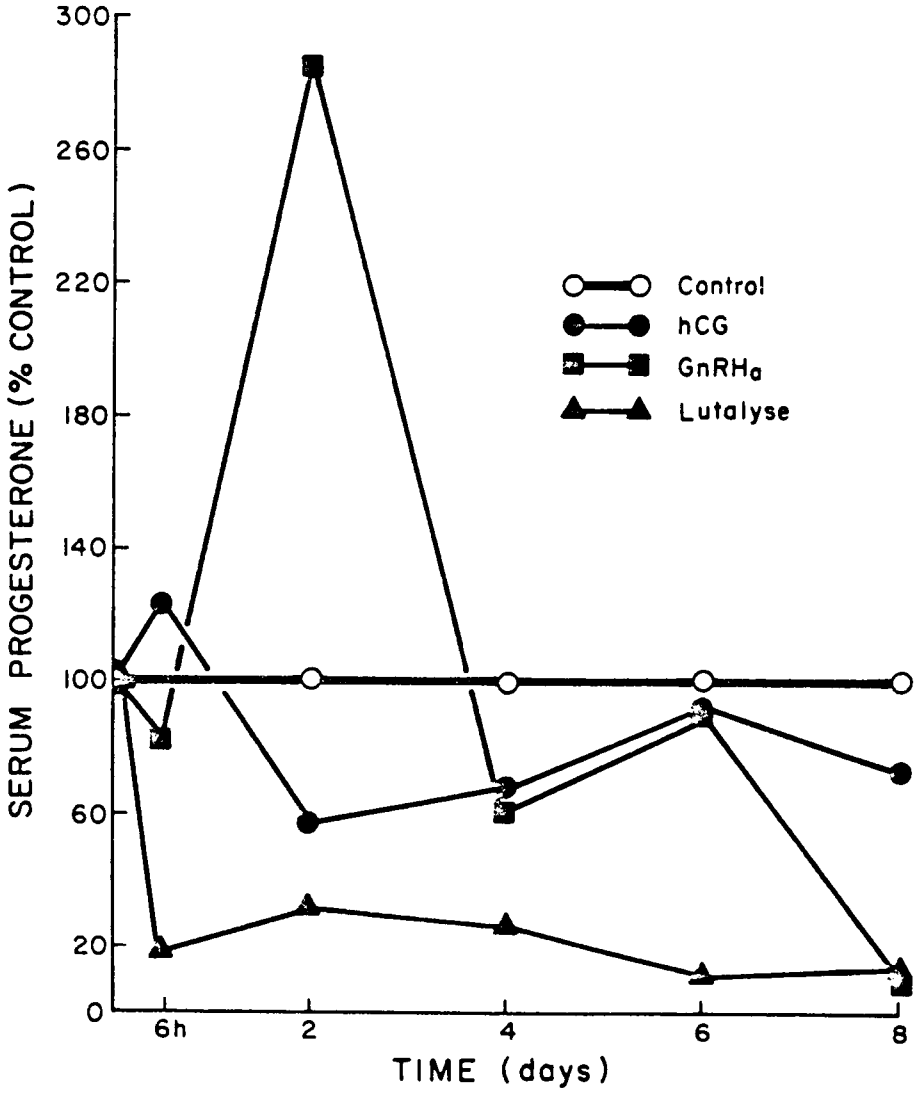
300
260
220
180
140
100
60
20
0
SERUM PROGESTERONE (% CONTROL)
Control
hCG
GnRHa
Lutalyse
6h
2
4
6
8
TIME (days)

Figure 11

3.7. The Effect of $PGF_{2\alpha}$, GnRH and hCG on the Morphological Characteristics of Rat Luteal Cells

Light and electron microscopy were used to observe the morphology of luteal cells from rats treated with $PGF_{2\alpha}$, GnRH and hCG. All figures shown illustrate cells from either the early (day 1 or 2) or later (day 4 or 5) time points after treatment.

Plates I-IV are light photomicrographs of rat luteal cells after hormone administration. In Plate I, figures A and B represent high power photomicrographs of luteal cells from control rats sacrificed at day 1 after treatment. The majority of cells are large and round with a fairly uniform shape and well defined nucleus and nucleolus. The cells are packed with large numbers of densely stained lipid droplets. Several smaller cells in which the cytoplasm and nucleus appear to stain more heavily are seen in figure A. These cells and their nuclei are more irregularly shaped compared to the majority of cells at this stage. They also appear to show very few lipid droplets. Figures C and D represent high and low power photomicrographs, respectively, of luteal cells from control rats sacrificed 5 days after treatment. The most noticeable change appears to be the decrease in the number of lipid droplets particularly in figure C. However, the vacuolated appearance of cells in figure D does suggest the presence of lipid droplets as cells may lose lipids during dehydration. At this later stage, the nuclei are heavily stained and the cell shape is highly irregular with numerous pseudopodia-like projections, characteristics that are very similar to those of the small, densely

Plate I. Light photomicrographs of luteal cells from control rats and stained with azure blue.

Figures A and B. High power light photomicrographs of luteal cells from control rats sacrificed at day 1. Dense staining lipid droplets (arrow). Small, densely stained cells (d) (x1250).

Figures C and D. Light photomicrographs of luteal cells from a control rat sacrificed at day 5.

Figure C. High power photomicrograph showing irregularly shaped cells with pseudopodia-like projections (arrow) (x1250).

Figure D. Low power photomicrograph showing vacuolated (v) appearance of cells (x500).

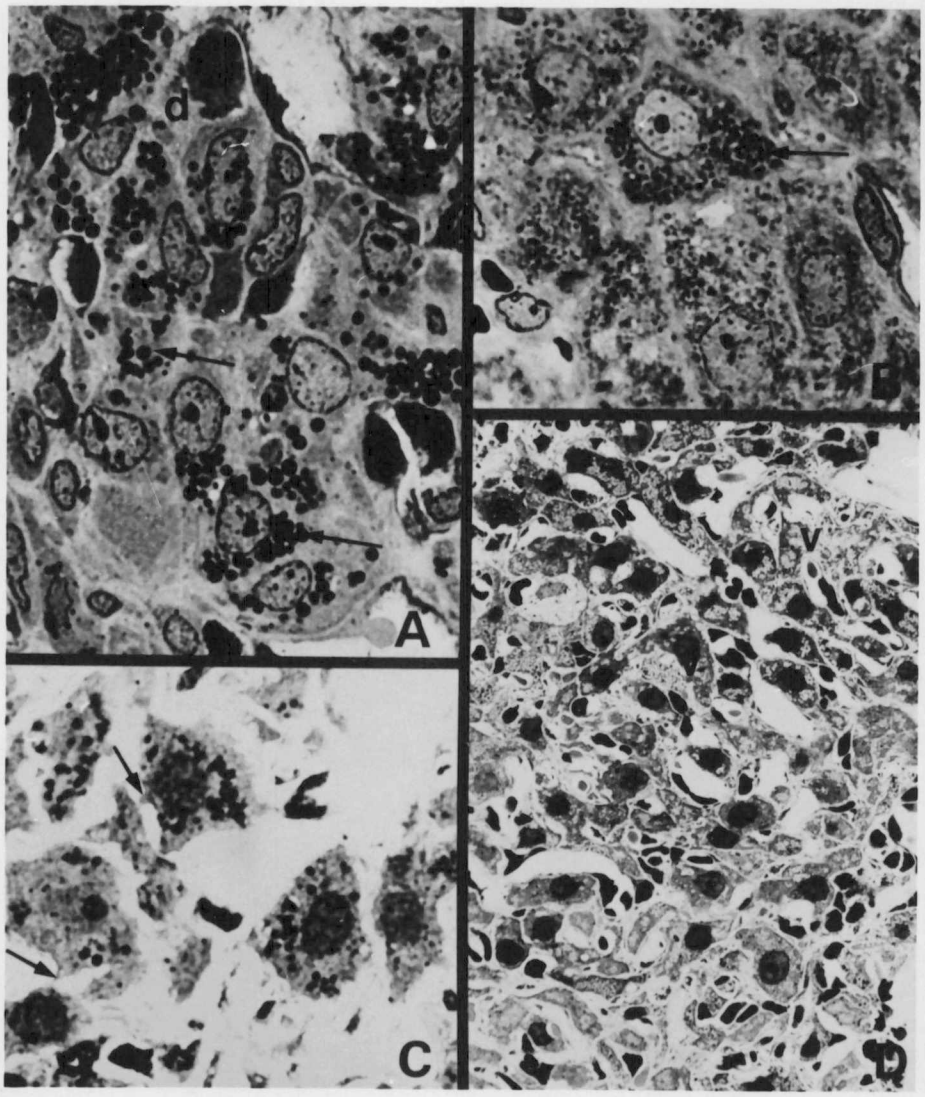

Plate I

Plate II. Light photomicrographs of luteal cells from rats treated with $PGF_{2\alpha}$ and stained with azure blue.

Figures A and B. High power light photomicrographs of luteal cells from $PGF_{2\alpha}$ treated rats sacrificed at day 1. Large lipid droplets (arrow), blood vessel (bv) (x1250).

Figures C and D. Lower power light photomicrographs of luteal cells from $PGF_{2\alpha}$ treated rats sacrificed at day 5. Large lipid droplets (arrow) macrophage (m), (x500).

Figure E. High power photomicrograph of luteal cells from a $PGF_{2\alpha}$ treated rat sacrificed at day 5. Large vacuolated (v) areas indicate the presence of lipid. During dehydration lipid may be lost (x1250).

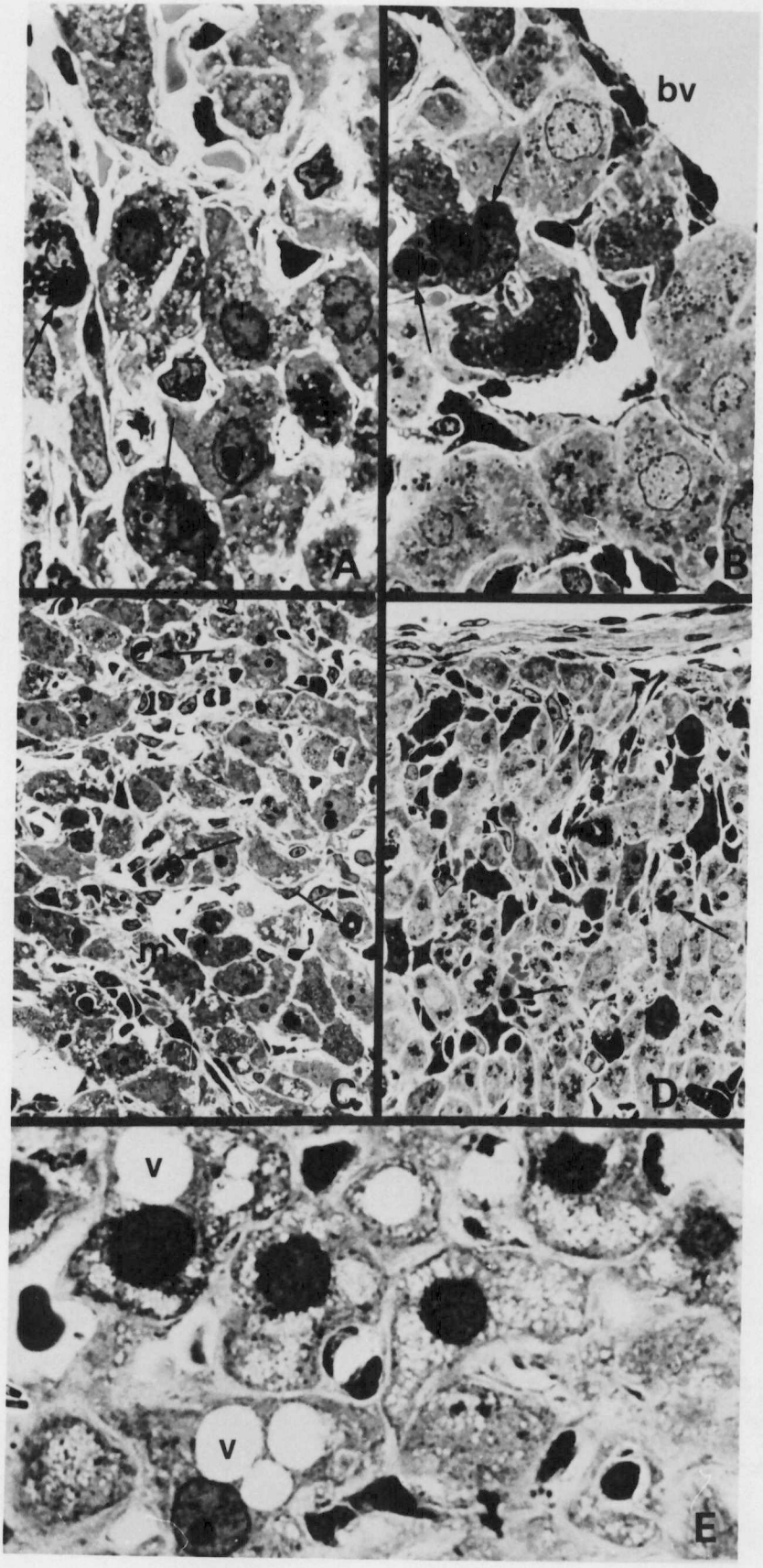

Plate II

Plate III. Light photomicrographs of luteal cells from rats treated with GnRH and stained with azure blue.

Figures A and B. Photomicrographs of luteal cells from rats treated with GnRH and sacrificed at day 1.

Figure A. Low power photomicrograph. Cells densely packed with lipid (arrow) (x500).

Figure B. High power photomicrograph. Large lipid droplets (L) (x1250).

Figures C and D. Low power photomicrographs of luteal cells from rats treated with GnRH and sacrificed at day 5. Heavily stained cells (D) have increased in number. Lipid droplets are numerous (arrow) (x500).

Figure E. High power photomicrograph of luteal cells from a rat treated with GnRH and sacrificed at day 5. Two cells (arrow) with large lipid droplets of various sizes (x1250).

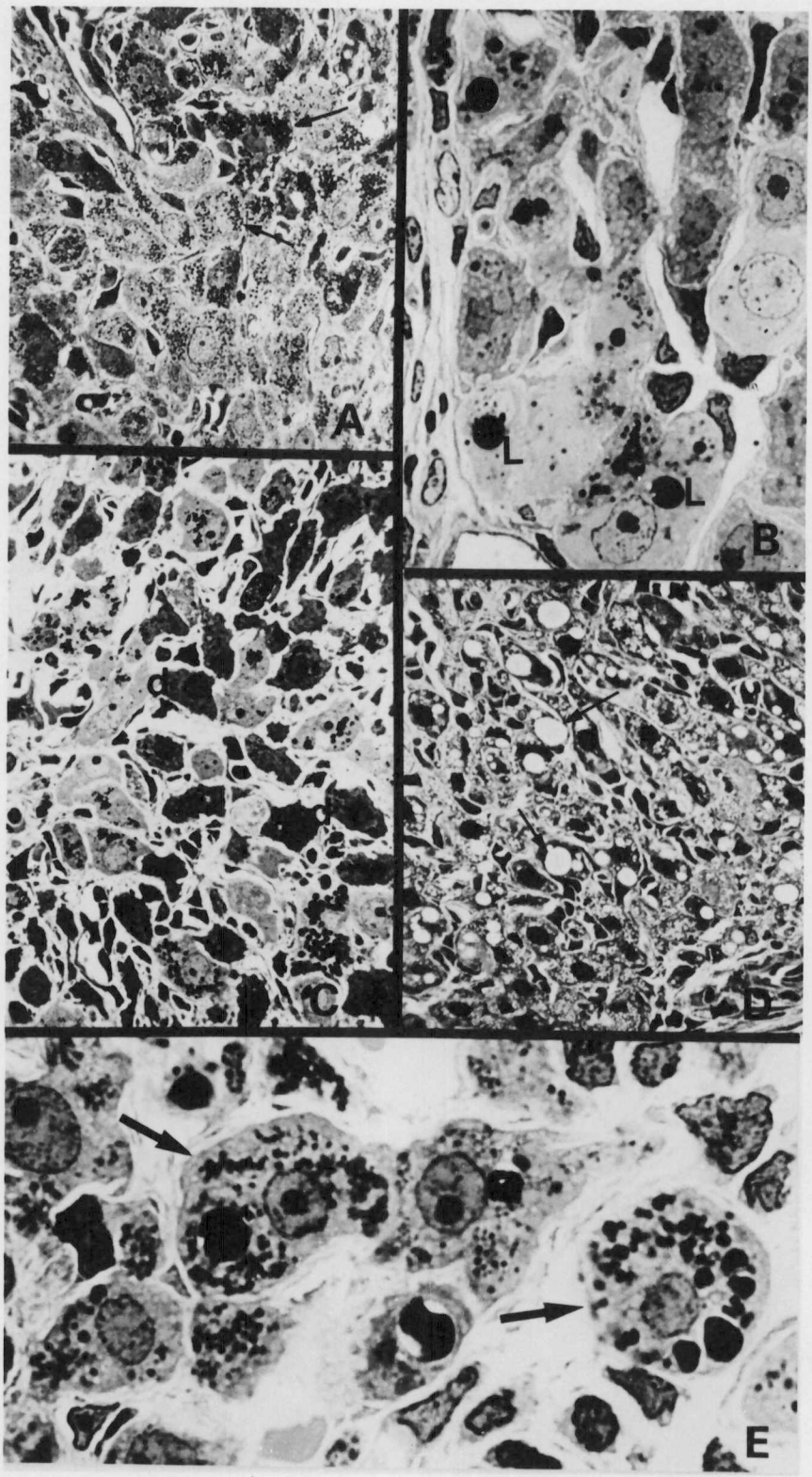

Plate III

Plate IV. Light photomicrographs of luteal cells from rats treated with hCG and stained with azure blue.

Figures A and B. Photomicrographs of luteal cells from rats treated with hCG and sacrificed at day 1.

Figure A. High power photomicrograph. Uniformly shaped cells (arrow) containing many lipid droplets (x1250).

Figure B. Low power photomicrograph showing both hyperchromatically and hypochromatically stained cells (x500).

Figures C and D. Photomicrographs of luteal cells from rats treated with hCG and sacrificed at day 5.

Figure C. High power photomicrograph. Several irregularly shaped, hyperchromatically stained cells (d) with numerous pseudopodia-like projections (arrow) (x1250).

Figure D. Low power photomicrograph. Dense staining cells increased in number (x500).

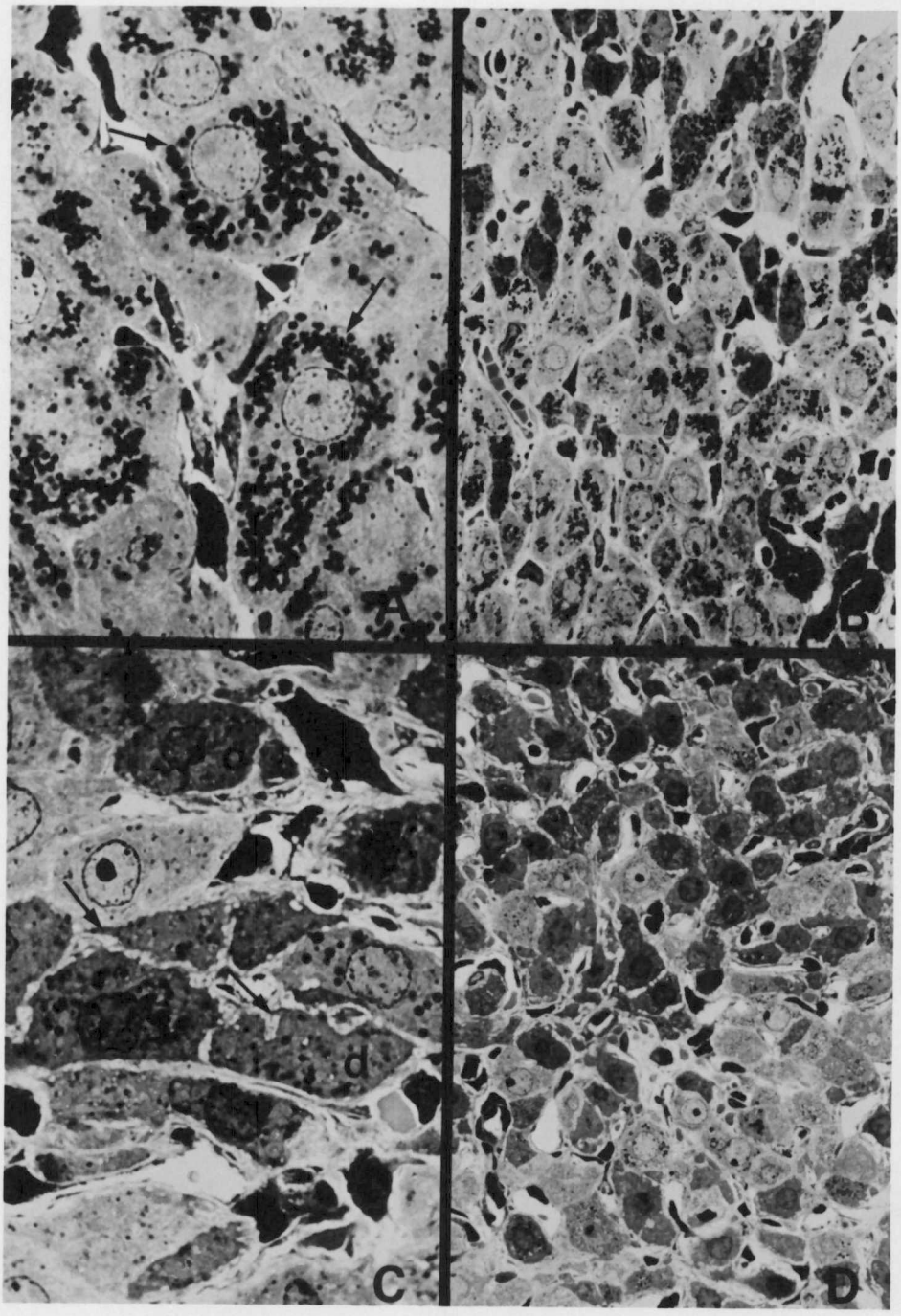

Plate IV

stained cells seen in figure A. A slight decrease in the size of these cells is also apparent.

In Plate II, figures A and B represent high power photomicrographs of luteal cells from rats at day 1 after treatment with $PGF_{2\alpha}$. The cells and their nuclei are more irregular in shape and appear to stain more deeply compared to cells from control rats at this time point. However, large uniformly rounded hypochromatically stained cells are still evident in figure B. The major difference between cells from control rats and those from $PGF_{2\alpha}$ treated rats is the decrease in the numbers of densely packed lipid droplets in the latter. Small vacuolated areas, however, are prominent in figure A. In some of the more heavily stained cells, however, there are large lipid droplets which result from the coalescence of many small lipid droplets. Figures C and D represent low power photomicrographs of luteal cells at day 5 after $PGF_{2\alpha}$ treatment. Densely stained cells appear to be more prominent at this time compared to the earlier time point. Very large lipid droplets associated with these cells are evident, although many hypochromatically stained cells still exhibit numerous small lipid droplets. A macrophage is also shown in figure C. Capillaries appear to be more narrow at day 5 compared to day 1 after $PGF_{2\alpha}$ treatment. This may be due to occlusion of blood vessels by endothelial cells. However, some blood vessels appear dilated which may indicate shrinkage of luteal cells or degeneration of the endothelial layer with a subsequent increase in the size of the capillary lumen. A high power photomicrograph of these later stage cells is shown in figure E. The size of these spaces suggests that

many smaller lipids have coalesced to form several large lipid droplets. The decrease in cell size and general disorganization of cells from rats treated with $PGF_{2\alpha}$ compared to cells from control rats was not apparent in light microscopic studies.

In Plate III, figures A and B represent cells from rats sacrificed one day after GnRH treatment. Figure A is a low power photomicrograph showing both hypochromatically and hyperchromatically stained cells containing large numbers of densely packed lipid droplets. Figure B is a high power photomicrograph showing several cells containing large lipid droplets similar to that observed in cells at day 1 in the $PGF_{2\alpha}$ treated group. In addition, the nuclei of these cells appear very similar to the irregularly shaped structures observed in cells from rats treated with $PGF_{2\alpha}$. In figures C and D which represent low power photomicrographs of cells at day 5 after GnRH treatment, there is an increase in the number of irregularly shaped, densely stained cells. Furthermore, the size of the lipid droplets has increased with coalescence of lipid droplets, more obvious in figure D. A high power photomicrograph (figure E) illustrates the nonuniformity in the size of lipid droplets.

In Plate IV, figures A and B represent high power and low power photomicrographs, respectively, of cells from rats one day after treatment with hCG. These cells resemble cells shown in figures A and B from control rats. Large numbers of densely packed lipid droplets surround the rather uniform hypochromatically stained nucleus. Fewer irregularly shaped, densely staining cells are shown in figure

B. At day 5 after hCG treatment, there is an increase in the number of densely stained cells as shown in figures C and D. The high power photomicrograph (figure C) illustrates pseudopodia-like structures originating from these cells. At this stage the nuclei appear to be irregularly shaped and there is a decrease in the number of lipid droplets. It appears that cells in figures C and D resemble cells from day 5 of control rats. Furthermore, features of these cells correspond closely to those observed in cells from the early time point in $PGF_{2\alpha}$ treated rats.

Plates V-VII are electron photomicrographs of luteal cells from rats treated with $PGF_{2\alpha}$, GnRH and hCG. In Plate V, figures A and B represent luteal cells from control rats one day after treatment. These cells contain many well defined elongated and spherical mitochondria which are characteristic of metabolically active cells. An extensive network of smooth and rough endoplasmic reticulum is evident. Lysosomal like structures are not evident at this stage but may become more prominent during regression of the corpus luteum. Figure C is a portion of a luteal cell from a control rat at day 5 after treatment. The mitochondria appear to be more uniformly spherical with less distinct cristae. These features are characteristic of cells with reduced activity. An increase in the size of lipid droplets suggests that the cell can accumulate cholesterol but is unable to convert it to progesterone. No autophagic vacuoles can be discerned. Figure D represents part of a luteal cell at day 1 after hCG treatment. The ultrastructure of this cell

Plate V. Electron photomicrographs of luteal cells from control rats and rats treated with hCG.

Figures A and B. Electron photomicrographs of luteal cells from control rats sacrificed at day 1. Mitochondria (M), rough and smooth endoplasmic reticulum (arrow), lipid droplets (L), nucleus (N), Figure A x10,000; Figure B x6000.

Figure C. Electron photomicrograph of a luteal cell from a control rat sacrificed at day 5. Mitochondria (M), rough endoplasmic reticulum (arrow), nucleus (N). Both heavily stained (L_1) and lightly stained (L_2) lipid droplets are present. A large lipid droplet (L_3) formed by fusion of several smaller lipid droplets (x10,000).

Figure D. Electron photomicrograph of a luteal cell from a rat treated with hCG and sacrificed one day later. Mitochondria (M), rough endoplasmic reticulum (arrow), dense staining lipid droplets (L) (x10,000).

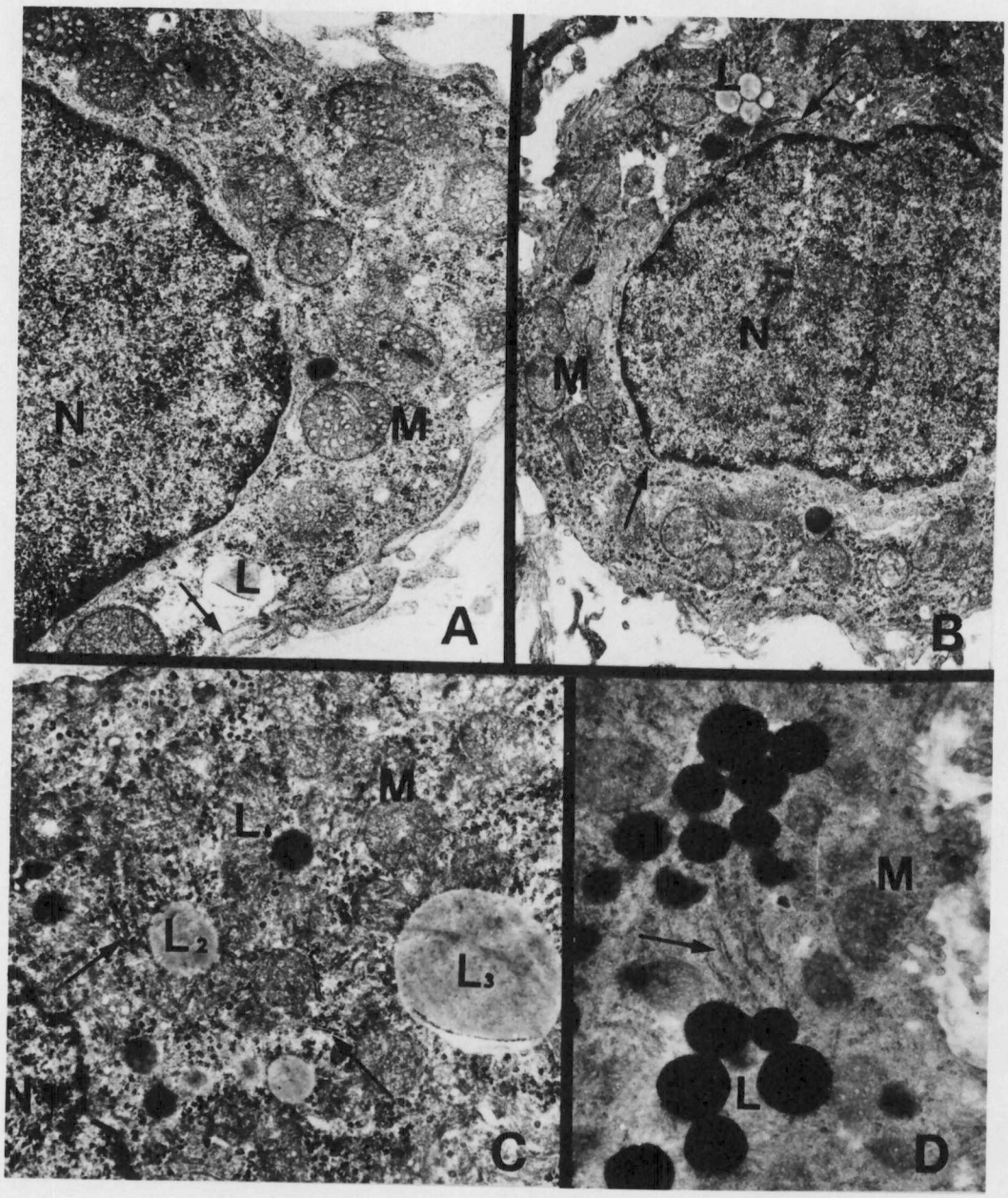

Plate V

Plate VI. Electron photomicrographs of luteal cells from rats treated with $PGF_{2\alpha}$.

Figures A and B. Electron photomicrographs of luteal cells from rats treated with $PGF_{2\alpha}$ and sacrificed at day 1. Mitochondria (M) Golgi body (G), nucleus (N), lipid droplets (L). A close association between two lipid droplets is seen in figure A (arrow) (Figure A x10,000; Figure B x12,000).

Figures C and D. Electron photomicrographs of luteal cells from rats treated with $PGF_{2\alpha}$ and sacrificed at day 5. Nucleus (N), lipid droplets (L). In figure C the lipid has been lost during dehydration (Figure C x8000; Figure D x6000).

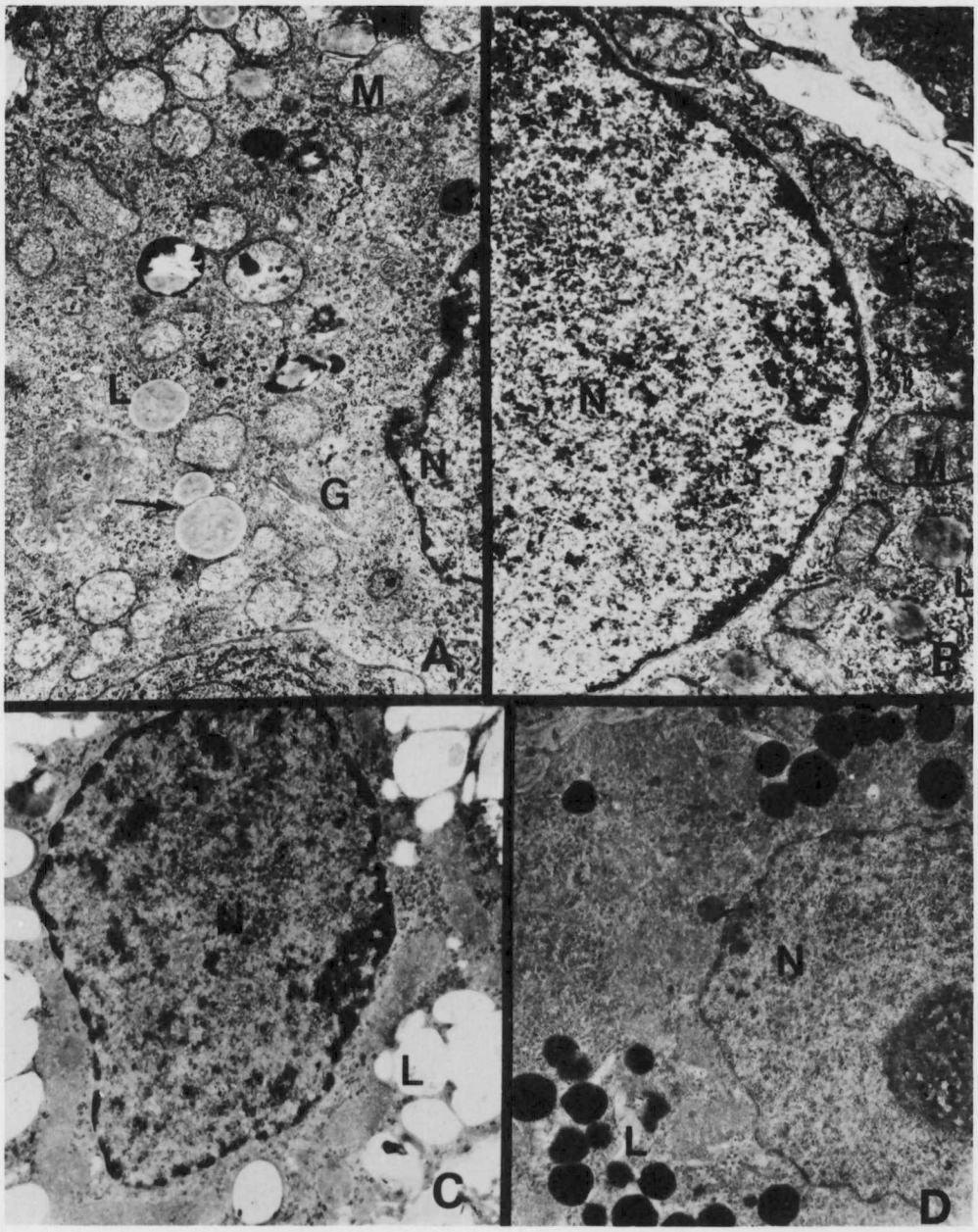

Plate VI

Plate VII. Photomicrographs of luteal cells from rats treated with GnRH.

Figures A and B. Electron photomicrographs of luteal cells from rats treated with GnRH and sacrificed at day 1. Mitochondria (M), lipid droplet (L), smooth endoplasmic reticulum (SER), Golgi body (G), nucleus (N). Several coated vesicles (arrow) are shown in figure A. A close association between lipid droplets and mitochondria is shown in figure A. (Figure A x6000; Figure B x4000).

Figures C and D. Electron photomicrographs of luteal cells from rats treated with GnRH and sacrificed at day 4. Mitochondria (M), nucleus (N). Several heavily stained lipid droplets are scattered between lightly stained lipids. In figure D several coated vesicles (arrow) are shown near the nuclear region (x15,000).

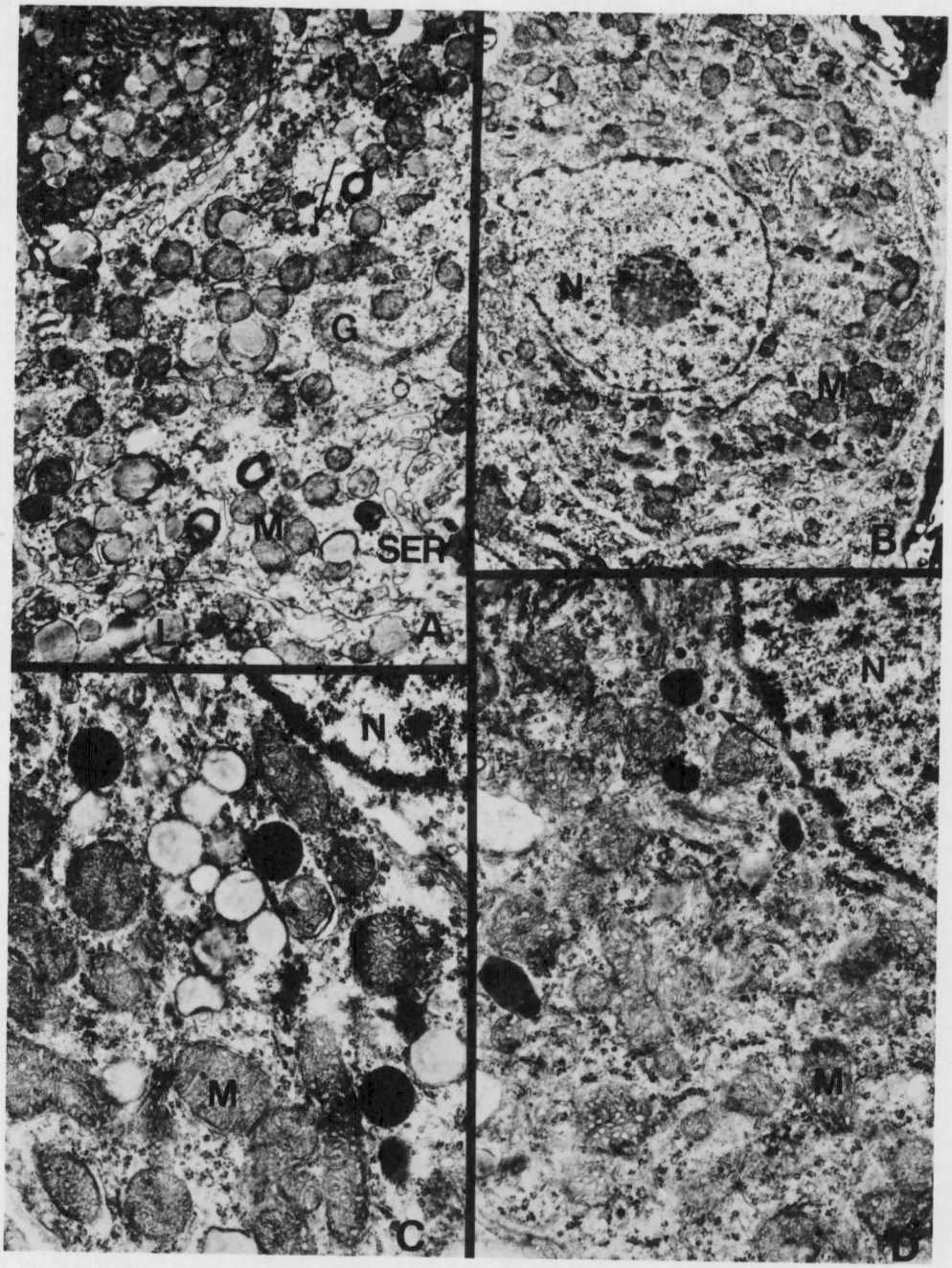

Plate VII

is similar to that observed in figures A and B. Many polymorphic mitochondria are present in addition to large numbers of densely stained lipid droplets surrounding the rough endoplasmic reticulum.

In Plate VI figures A and B are of luteal cells obtained from rats at day 1 after $PGF_{2\alpha}$ treatment. In figure B the mitochondria are both spherical and elongated. However, in figure A the mitochondria appear less uniform in appearance. The cristae are not as distinct and the general appearance of the mitochondria is one of disorganization when compared to mitochondria from cells of control rats at this stage. An apparent clumping of the mitochondria is shown in figure A. The lipid droplets appear to be more numerous although less uniform in size compared to control cells at day 1. A close association between two lipid droplets is shown in figure A and may indicate fusion prior to the formation of a large lipid droplet. The smooth endoplasmic reticulum is still evident although it appears vesicular in certain regions of the cell. Figures C and D represent portions of luteal cells at day 5 after $PGF_{2\alpha}$ treatment. The most obvious change is the decrease in the number of distinct mitochondria and the accumulation of large numbers of lipid droplets, many of which have coalesced. The irregular shape of the nucleus at this stage is clearly seen in figure C.

In Plate VII figures A and B show portions of luteal cells at day 1 after treatment of rats with GnRH. Many spherical mitochondria are apparent and surround several elongated mitochondria.

A close association between several mitochondria and lipid droplets is shown in figure A. Numerous lipid droplets are shown along with an extensive network of smooth and rough endoplasmic reticulum. However, in figure A the smooth endoplasmic reticulum does show a rather vesicular appearance. In figures C and D, which represent cells from rats sacrificed at the later time point, the mitochondria appear to be less well defined with indistinct, rather vesicular cristae. Although these electron photomicrographs do not show the exaggerated lipid droplet shown with the light microscope (Plate III, figures C and D), several lipid droplets do exhibit a close apposition with one another prior to fusion. In figure D several small, densely stained bodies are shown near the nucleus. These are coated vesicles which are characterized by a halo of clathrin protein. The smooth endoplasmic reticulum is not as evident as in figures A and B although an extensive array of rough endoplasmic reticulum is apparent.

4. Discussion

The effect of hCG, GnRH and $PGF_{2\alpha}$ on LH and PRL receptor content in the rat corpus luteum was examined over a period of eight days following treatment. Results of these investigations demonstrated an initial reduction in LH receptors which was greatest in rats treated with a desensitizing dose of hCG. Similar observations have also been reported by Conti et al. (1979b) after hCG treatment. This phenomenon may be one way by which target cells reduce their

sensitivity to prevent overstimulation. In rats that were treated with a GnRH analogue the initial decrease in LH receptors may be partially due to LH released from the pituitary under GnRH stimulation. GnRH is known to stimulate the release of LH by the pituitary with a consequent decrease in LH receptors in the ovary (Reddy et al., 1980; Kledzik et al., 1979b). The action of GnRH in reducing ovarian LH receptors through stimulating LH release by the pituitary is analogous to exposing the ovary to high concentrations of exogenous hCG. However, Clayton et al. (1979) demonstrated the presence of receptors for GnRH in the rat luteal cell. They also report GnRH induces a decrease in ovarian LH receptors in PMSG/hCG primed, hypophysectomized rats, suggesting that GnRH can exert a direct inhibitory action on the ovary. The initial reduction in LH receptors after $PGF_{2\alpha}$ treatment may reflect a partial luteolytic effect since $PGF_{2\alpha}$ is widely recognized as the luteolytic agent in the rat. Although evidence for $PGF_{2\alpha}$ stimulation of LH release from the pituitary is limited, several investigators have reported an increase in serum LH after $PGF_{2\alpha}$ treatment in several species (Warberg et al., 1976; Bono et al., 1980). It is possible that the action of $PGF_{2\alpha}$ is similar to that of GnRH in reducing ovarian LH receptors. It would be reasonable to expect that the amount of LH released by the pituitary into the circulation after either GnRH or $PGF_{2\alpha}$ administration is much less than hCG administered exogenously. This could explain the smaller decease in LH receptors over days 1 and 2 compared to the effect of hCG. In experiment II the pattern of LH receptor

recovery after the initial decrease by $PGF_{2\alpha}$ is also suggestive of an effect at the level of the pituitary. However, the possibility that the dose given was not large enough to cause a prolonged loss of receptors should not be dismissed. In experiment III, a large dose of $PGF_{2\alpha}$ did cause an irreversible loss of LH receptors. The luteolytic effect is probably mediated by receptors for $PGF_{2\alpha}$ present on the ovary (Wright et al., 1980).

The early decrease in LH receptors after hCG and GnRH treatment was followed by full recovery within 2-3 days. This recovery may involve the synthesis and insertion of new receptors or the recycling of old receptors. Interestingly, in rats treated with 1mg of $PGF_{2\alpha}$, the loss of LH receptors was followed by a return to control levels. This was unexpected since $PGF_{2\alpha}$ is known to cause an irreversible loss in LH receptors. However, this result may reflect the lower dosage used in this experiment compared to a second experiment in which rats were treated with 2mg $PGF_{2\alpha}$. Additionally, the pattern of receptor decrease and reversibility was also demonstrated in a dose-response experiment where at the highest dose of 2mg of $PGF_{2\alpha}$ LH receptors returned to control values.

A similar pattern of loss and recovery was also evident for PRL receptors after treatment with hCG, GnRH and $PGF_{2\alpha}$. The initial reduction in PRL receptors was more pronounced compared to LH receptors but the recovery occurred earlier. Several investigators have also shown a concomitant decrease in both PRL and LH receptors after gonadotropin treatment in both the testis (Chan et al., 1981)

and the ovary (Davies et al., 1980; Chen et al., 1981). In addition, Davies et al. (1980) reported an earlier recovery of PRL receptors compared to a more sustained reduction in LH receptors. A coordinate reduction in both LH and PRL receptors in the ovary has, furthermore, been demonstrated after GnRH (Harwood et al., 1980d) and $PGF_{2\alpha}$ (Behrman et al., 1978) treatment. The recovery of PRL receptors was in marked contrast to the prolonged loss in LH receptors observed after treatment with 2mg of $PGF_{2\alpha}$. However, since ^{125}I-hGH binds to both PRL and GH receptors, the recovery may reflect an increase in binding to GH receptors rather than PRL receptors. The similar pattern of decrease in receptors for PRL and LH after treatment with hCG, GnRH or $PGF_{2\alpha}$ suggests that down regulation of these receptors is coordinated. Although a differential rate of recovery was observed for both receptors the possibility of a close physical association between LH and PRL receptors during down regulation should not be overlooked.

To determine the functional state of the corpus luteum after treatment with hCG, GnRH and $PGF_{2\alpha}$, serum progesterone levels were measured. hCG caused an initial increase in progesterone followed by a prolonged refractory period. The initial increase in progesterone levels after hCG treatment contrasts with the marked decrease in LH receptors observed at this time. However, the rise in progesterone was not great and may simply reflect a brief exposure to a large dose of hCG. A sustained decrease in serum progesterone levels is a result of a more prolonged decrease in LH receptors. The effect of

GnRH on serum progesterone may involve an initial direct inhibitory action on the ovary as indicated by a small decrease within one day. The apparent increase observed at this time in experiment II possibly masks an earlier, transient reduction in steroid levels since the first time point examined was at day 1. On the other hand, a marked elevation of progesterone levels observed soon after the initial inhibitory response suggests a secondary action of GnRH in stimulating the release of LH from the pituitary. High levels of LH would in turn increase serum concentrations of progesterone. The fact that the increase in serum progesterone is higher in rats treated with GnRH compared to those treated with hCG may be a consequence of a continuous increase in circulating levels of LH caused by GnRH stimulation of the pituitary in contrast to a rapid rise following administration of exogenous hCG. High levels of hCG may rapidly saturate all the available receptor sites causing an initial increase in progesterone production but at the same time stimulating rapid down regulation of receptors for LH. The loss of LH receptors, both occupied and unoccupied, may involve clustering of receptors and endocytosis within small vesicles (Catt et al., 1979b). The increase in mobility of LH/hCG bound receptors through clustering and internalization may result in a concomitant decrease in other receptors on the cell surface. By contrast, GnRH stimulates LH release from the pituitary gland so that the concentration of circulating gonadotropins gradually increases. The initial high levels of progesterone after GnRH stimulation may thus be a consequence of high levels of LH

which are maintained over a longer period of time. Since many more unoccupied receptors are present on the cell surface for a longer period of time to bind additional LH, progesterone levels continue to increase compared to a more rapid desensitizing effect observed with high levels of hCG. However, as more receptors become occupied, with subsequent down regulation, the concentration of progesterone falls. The marked stimulatory effect of GnRH shown in these experiments contrasts with the inhibitory effects of progesterone production demonstrated by other investigators (Pieper et al., 1980; McDonald and Beattie, 1979; Hsueh and Erickson, 1979a). However, a transient desensitizing effect on progesterone levels was evident within one day in both hCG and GnRH treated rats. In addition, the rapid drop in progesterone production from day 2 in GnRH treated rats was followed by gradual recovery to control levels. This pattern was also observed in hCG treated rats although in experiment II progesterone levels remained well below control values for the entire, 5 day period.

The effect of $PGF_{2\alpha}$ in causing a rapid and sustained decrease in progesterone contrasts to the transient changes demonstrated in LH and PRL receptors. The luteolytic effect in terms of a rapid and prolonged decrease in progesterone production was more evident at the higher dose. Similar results have been reported by Grinwich et al. (1976) and Torjesen et al. (1978a). In the three experiments the effect of hCG, GnRH and $PGF_{2\alpha}$ on serum progesterone levels are in close agreement with one another. The results of these studies

suggest that $PGF_{2\alpha}$ exerts a luteolytic effect with a prolonged decrease in serum progesterone while hCG and GnRH promote a desensitizing effect without a sustainable reduction in progesterone levels.

The actions of hCG, GnRH and $PGF_{2\alpha}$ share several common features with respect to changes in receptor and progesterone levels. However, the present studies demonstrate a distinct effect of $PGF_{2\alpha}$ in promoting an irreversible decrease in progesterone while hCG and GnRH cause both receptor and progesterone levels to return to normal before decreasing.

The effect of hCG, GnRH and $PGF_{2\alpha}$ on the morphology of the corpus luteum was examined at both the light microscopic and electron microscopic levels. At the light microscopic level luteal cells from both control and hCG treated groups were similar in appearance to one another during the early stage after treatment. These cells were actively involved in the synthesis of progesterone as indicated by the presence of large numbers of dense staining lipid droplets. Although the accumulation of lipid in the form of cholesterol is an indication that progesterone synthesis is retarded, the presence of large numbers of lipid droplets may also indicate that the cells are accumulating cholesterol at a faster rate than is being converted to progesterone. However, it was interesting to observe several very large lipid droplets in cells from GnRH and $PFG_{2\alpha}$ treated rats. These structures were prominent in smaller, more heavily stained cells, particularly in rats treated with $PGF_{2\alpha}$, and probably resulted from fusion of smaller lipid droplets. The presence of large lipid

droplets indicates that cells are unable to convert cholesterol to progesterone with the result that cholesterol ester accumulates.

In the $PGF_{2\alpha}$ treated group, small vacuolated areas were observed which may indicate aging of the cells. Paavola (1979) has suggested that older cells contain lipid which is more easily extracted during processing of the tissue, compared to younger cells. By contrast, however, Rennels (1966) found that preservation of lipid was much better in luteal cells that were inactive compared to cells that were actively synthesizing progesterone. This vacuolated appearance was also very prominent in the GnRH treated group at day 5 after treatment. The presence of very large vacuoles at this time in both GnRH and $PGF_{2\alpha}$ treated groups indicates fusion of lipid droplets and the inability of these cells to synthesize progesterone. Furthermore, the presence of small vacuolated areas shown in control cells at day 5 probably indicates aging of cells prior to luteolysis. Aging of these cells may also involve a decrease in the number of lipid droplets. The cells appear irregular in shape and generally are less well defined compared to cells at day 1 after treatment. It is therefore interesting to speculate on a premature aging effect on luteal cells by $PGF_{2\alpha}$

Identification of heavily stained cells, which appear to increase in number from day 1 to day 5 after treatment and may represent a second population of luteal cells, suggests that they are aged cells which can develop early, depending on the type of hormonal stimulation. The irregular shape of these cells and the presence of

pseudopodia-like projections compare favorably with older luteal cells shown at day 5 in the control and hCG treated group. It is possible that those cells in the $PGF_{2\alpha}$ (or GnRH) treated groups which are heavily stained and contain large lipid droplets or smaller vacuolated areas are prematurely aging cells in which these hormones have accelerated the process. Interestingly, numerous investigators have identified the presence of several populations of cells in the corpus luteum. Fitz et al. (1982) and Koos and Hansel (1981) characterized, respectively, two cell types in the sheep and cow. In the absence of LH larger cells produced more progesterone than smaller cells but were unable to produce progesterone in the presence of LH. Furthermore, larger cells contained few LH receptors but more receptors for $PGF_{2\alpha}$ and PGE_2 compared to smaller cells.

Cells that are actively synthesizing progesterone are characterized ultrastructurally by the presence of many elongated mitochondria with lamelliform cristae and an extensive network of smooth endoplasmic reticulum. In addition, luteinized cells exhibit little accumulation of lipid droplets which indicates that the cells are actively involved in the conversion of cholesterol ester to steroid. In studies involving the effect of hCG, GnRH and $PGF_{2\alpha}$ on the ultrastructure of luteal cells at day 1, few differences could be discerned between different treatment groups. Mitochondria appear, for the most part, spherical in shape although several elongated structures are shown. As cells become more active in synthesizing progesterone there is a change in the repertoire of enzymes which may

alter the structure of the mitochondria (Long, 1973). In cells from both GnRH and $PGF_{2\alpha}$ treated groups there appears to be an increase in the number of lipid droplets at day 1 compared to cells from control rats. In addition, in the $PGF_{2\alpha}$ group, although not well demonstrated at this stage, several lipid droplets are in the process of apparently fusing to form a larger droplet. This feature is more obvious at day 5 in these cells and is a characteristic of cells that are unable to convert cholesterol to progesterone. These cells also appear to show lysosomal-like structures and rather ill-defined mitochondria even at this early stage after treatment. In cells treated with GnRH an intimate association between mitochondria and lipid droplets is seen at day 1 after treatment. This phenomenon may be a characteristic of cells undergoing reduced steroidogenesis since Rennels (1966) found it to be a feature of inactive luteal cells obtained from hypophysectomized rats. If a close association between lipid and mitochondria is characteristic of cells not actively synthesizing and secreting progesterone one would expect that cells from rats treated with $PGF_{2\alpha}$ would contain many such features. However, this was not observed at either the early or late time points examined. Lysosomal-like structures are also present in cells from GnRH treated rats.

Ultrastructural characteristics of luteal cells at days 1 and 5 treated with GnRH or $PGF_{2\alpha}$ appear to be consistent with those of inactive luteal cells described by Rennels (1966) and Long (1973). It is possible that $PGF_{2\alpha}$ and GnRH initiate structural changes in the luteal cell with a subsequent decrease in progesterone production.

However, it is generally held that progesterone levels change before any change in morphology occurs. Since the initial decrease in progesterone in rats treated with GnRH was transient compared to the irreversible decreases caused by $PGF_{2\alpha}$, fewer demonstrable changes in the ultrastructure would be expected. However, the pattern of progesterone levels observed in GnRH treated rats clearly resembles the pattern produced by hCG. It would be reasonable then to expect that the morphology of luteal cells from GnRH treated rats would be similar to cells from hCG rather than $PGF_{2\alpha}$ treated rats. One must bear in mind, however, that the time points at which the tissues were examined microscopically were day 1 which corresponds to the initial decrease in progesterone in $PGF_{2\alpha}$, and GnRH groups and day 5 in which steroid levels were dramatically reduced in these groups compared to rats treated with hCG.

The present series of experiments demonstrate the effect of hCG, GnRH and $PGF_{2\alpha}$ on LH and PRL receptor progesterone levels and on the morphology of the corpus luteum in the superovulated rat. Although many similarities were observed between the effect of GnRH and the other two hormones, it is difficult to conclusively determine a luteolytic or a desensitizing role for GnRH.

CHAPTER III

FLUORESCENCE LOCALIZATION OF hCG AND PRL BINDING IN VITRO

1. Introduction

Receptors for both LH and PRL are located on the surface of the luteal cell. Binding of specific hormones to these receptors initiates clustering of receptors on the cell surface, followed by internalization of the hormone-receptor complexes. Results of studies shown in the previous chapter demonstrate a loss of both LH and PRL receptors during desensitization. Based on these findings it would be expected that LH (or hCG) binds to its receptor and initiates clustering and internalization of receptors for LH and PRL.

Radiolabelling techniques have been used extensively to quantitate and characterize LH and PRL receptors in the ovary. However, visual identification by a variety of fluorescent staining techniques provides a more direct method for the localization of receptors. Since peptide hormones bind to their receptors on the plasma membrane, studies involving fluorescently labelled, biologically active conjugates of the hormones have demonstrated hormone uptake with subsequent internalization and processing of the hormone-receptor complex. Fluorescence labelling of epidermal growth factor (EGF) (Haigler et al., 1978) and thyroid stimulating hormone (TSH) (Avivi et al., 1982) has provided direct visualization of hormone binding followed by endocytosis of the hormone (and its receptor).

The purpose of this study was to localize receptors for LH and PRL in the rat luteal cell. Purified preparations of fluorescein isothiocyanate (FITC) conjugated to hCG and tetramethylrhodamine isothiocyanate (TRITC) conjugated to oPRL were used as specific markers for the uptake of hCG and oPRL, respectively. The FITC-hCG conjugate allows for the localization of hCG uptake in luteal cells while the TRITC-oPRL conjugate permits visualization of the uptake of PRL. Since the maximum absorption of FITC occurs at a wavelength of 489nm while TRITC absorbs maximally at 554nm it was possible to use both fluorochromes at the same time to study the differential distribution of the uptake of two different protein hormones. This phenomenon is of particular advantage in a system where it is necessary to study two specific features at the same time.

In order to achieve the aim of this study it was necessary to purify the resulting conjugates to remove remaining unbound FITC and TRITC which can cause a high degree of nonspecific staining. In addition further purification procedures were carried out to remove unwanted proteins from the fluorochrome-hormone conjugates.

2. Materials and Methods

2.1. hCG-Desensitization of Cultured Rat Granulosa-lutein cells

a. Granulosa-lutein cell culture. Fourteen rats were primed with 50 IU of PMSG and sacrificed 48 hours later. Ovaries were removed, trimmed free of fat and connective tissue and placed in a

clean scintillation vial containing 10ml of sterilized Eagle's-Ham's media (see Appendix A) with 10% fetal calf serum (Gibco Lab, Grand Island, New York). The following procedures were then carried out under sterile conditions. Ovaries were removed from the vial and placed in a sterile glass petri dish containing a small amount of media. Tissues were minced using a razor blade and the material pipetted into a 10ml Falcon screw top tube (Fisher). Additional media was pipetted into the petri dish and remaining tissue removed and added to the 10ml Falcon tubes. A pasteur pipette was used to break up clumps of cells by pipetting up and down several times. The cell suspension was allowed to sit for one minute in these tubes. The supernatant containing granulosa cells was removed and pipetted into 50ml Corning screw top centrifuge tubes (Fisher). Several milliliters of media were added to the original Falcon tubes and again the suspension was allowed to sit for one minute. The supernatant was pipetted into the Corning centrifuge tubes. This procedure was repeated several more times until most of the granulosa cells were removed from the original tissue suspension. Fifty milliliter tubes containing the cell suspension were centrifuged (IEC HN-S11 Centrifuge) at 1000 rpm for 5 minutes and the supernatant decanted. The pellet was resuspended in 10ml of media with thorough pipetting to remove large cell clumps. A drop of the cell suspension was placed on a hemocytometer and the number of cells counted. Appropriate dilution of the cell suspension was carried out with media. One milliliter of cell suspension (1×10^6 cells/ml) was added to 35 x 10mm Falcon 1008

petri dishes (Becton, Dickinson and Co., Maryland) containing plastic cover slips. Trypan blue was used to test for cell viability and cells were incubated at 37°C (5% CO_2, 95% air).

b. Progesterone and estrogen levels in media containing cells treated with hCG. Cells were incubated over a period of 7 days. Cultures were photographed periodically using an Olympus inverted microscope (200x; green filter). Culture media was changed every day and replaced with fresh media containing 10% fetal calf serum. At days 2 and 3 after plating, media was collected from six dishes prior to the addition of hCG and stored at -20°C until assayed for progesterone and estrogen. hCG (2570IU/mg) was prepared by dissolving 1mg of hCG in 1ml of PBS. One hundred micrograms of sterile hCG solution were added to a bottle containing 100ml of media (Eagle's-Ham's with 10% fetal calf serum). On day 3 after plating 1ml of media containing hCG (1µg/ml) was added to 11 dishes while the control group received media without hCG. From day 4 onwards media was changed in all dishes but was collected and stored at -20°C from the previous six dishes (three control and three treatment dishes) only. In addition, from day 4 after plating, three dishes from both treatment and control groups were removed and stored at -20°C until cells were assayed for LH receptors.

Spent media was diluted 1:10 with PBS and progesterone and estrogen levels determined in triplicate by radioimmunoassay. The procedures were similar to the method described previously in Chapter II (section 2.7, part b) with the following notable exceptions. For

the standard curve radioiodinated progesterone (progesterone-3-(o-carboxymethyl)oximino-2-[^{125}I]iodohistamine; Amersham) was used in place of tritiated progesterone and was counted in a γ counter (Beckman 4000) rather than a liquid scintillation counter. Estrogen levels in cell culture media were also analyzed using the procedure described earlier in Chapter II (section 2.8).

c. LH receptors in cultured rat granulosa-lutein cells treated with hCG. Frozen cells were thawed and 1.5ml of 0.1% BSA/PBS was added to all dishes. An excess of unlabelled hCG was added to several dishes to monitor nonspecific binding. After 5 minutes, 100µl of 1.6 x 10^6cpm of radiolabelled hCG was added to all dishes. Incubation was carried out at room temperature for 17 hours. After overnight incubation the media containing unbound radiolabelled hCG was aspirated from each dish into culture (12 x 75mm) tubes. Three milliliters of 0.1% BSA/PBS was added to all dishes. After 5 minutes the solution was again transferred to the tubes containing the original media. The solution in the tubes was centrifuged at 3000 rpm for 30 minutes in a Beckman tabletop centrifuge and the supernatant was aspirated leaving a small pellet consisting of cells that were originally detached from the coverslip. Cells attached to the cover slip and petri dish were scraped off with a rubber policeman and pipetted into the original culture tubes containing the small pellet. The tubes were again centrifuged at 3000 rpm for 30 minutes followed by aspiration of the supernatant. This procedure was repeated once more and the final pellet was counted for radioactivity.

2.2. Preparation and Purification of Fluorochrome Conjugates

a. Coupling of fluorescein isothiocyanate (FITC) with human chorionic gonadotropin (hCG) and purification of conjugate.

Conjugation of FITC and hCG. FITC was coupled to hCG by modification of a procedure described by Dizerega et al. (1980). FITC (0.25mg), obtained from Research Organics Inc. (Cleveland, Ohio), was dissolved in 500µl of 0.1M Na_2 HPO_4 solution in a Wheaton Omnivial. In a separate Omnivial 5mg of hCG (Sigma; 2570IU/mg) was dissolved in 2.7ml of PBS (pH 7.4) and the solution transferred to a clean glass scintillation vial. Fifty microliters of PBS containing approximately 3000cpm of ^{125}I-hCG to monitor protein recovery was added to the vial. To the vial containing hCG, 540µl of 0.2M Na_2PO_4 was added and the solution was stirred gently. FITC solution was added dropwise to the solution of hCG so that the molar ratio was 5 (FITC) : 1 (hCG). The pH was adjusted to 9.3 with 0.1M Na_3PO_4 and 500µl of 0.145M NaCl was added to the mixture. The vial was covered with foil and the reaction was allowed to proceed at 4°C with continuous stirring on ice for two hours. The pH was periodically adjusted with 0.1M Na_3PO_4 to approximately 9.3. After two hours the foil covered solution was allowed to incubate overnight at 4°C with continuous stirring. After overnight incubation the solution containing FITC coupled to hCG (FITC-hCG) was transferred into a 15cm length piece of dialysis tubing (Spectral Medical Ind. Inc., Los Angeles) (0.5cm diameter; 10,000 molecular weight cut off) tied at one end. The

solution was concentrated overnight under vacuum in a 500ml flask at 4°C. After vacuum concentration, 1ml of the remaining solution was allowed to dialyse overnight in a beaker containing PBS (pH 7.4).

Separation of bound FITC from unbound FITC. The FITC-hCG conjugate was separated from free FITC by column chromatography using a Sephadex G-25 column (10ml) equilibrated with PBS. Fractions (1ml) were collected by eluting the column with PBS. Both the FITC-hCG and free FITC bands could easily be distinguished on the column. Fluorescein and protein concentrations were determined in each fraction by using spectrophotometric absorbance (Perkin Elmer Lambda 3 uv/vis) at 495nm and 280nm, respectively. In addition, all fractions were counted for radioactivity (Beckman Gamma 4000) to identify the hCG peak. Fractions that contained the highest levels of radioactivity and absorbance readings at both 280nm and 495nm were selected and pooled together. The pooled fraction was again vacuum concentrated overnight as previously described and later dialysed for 24 hours in PBS (pH 7.4).

Preparation of the Sephadex G-75 column. A Sephadex suspension was prepared by dissolving approximately 40 grams of Sephadex G-75 in distilled water. Over a period of several days the supernatant was decanted and deaeration carried out under vacuum according to manufacturer's recommendations. Fine particles suspended in the supernatant were removed by final aspiration of the supernatant and the beads were again resuspended in distilled water. The Sephadex suspension was carefully poured into a 90 x 2.5cm column

and allowed to settle under gravity. PBS was added to the reservoir and the column allowed to equilibrate with 400mls of PBS. During this time the column was adjusted to a flow rate of approximately 6ml/min with a maximum operating pressure of 100cm.

Calibration of a Sephadex G-75 column. Prior to the addition of standard protein solutions to the column the void volume and column volume were determined using a solution of blue dextran (Sigma) and FITC. Solutions of blue dextran (5mg/ml) and FITC (1mg/ml) were prepared in PBS. One half milliliter of each solution was mixed thoroughly and several drops of 2M sucrose were added to the solution. Prior to addition of the mixture to the column, PBS was removed from the top of the column. The blue dextran-FITC solution was carefully pipetted on top of the gel using an 8cm plastic catheter tube attached to an 18 gauge needle and 5ml syringe. The column was unclamped and the eluent collected in a 50ml measuring cylinder. The volume of buffer collected prior to elution of both dextran and FITC was recorded. The void volume (v_o) was designated as that volume of buffer collected prior to elution of blue dextran. The column was re-equilibrated with 400mls of PBS to remove contamination.

To determine the elution fraction expected to contain the fluorescein conjugate, a number of standard protein solutions were eluted from the column. The standard proteins (Sigma, MW-GF-200) chosen were ovalbumin (molecular weight = 45,000), chymotrypsinogen A molecular weight = 25,000) and aldolase (molecular weight = 158,000).

The standard protein solution consists of 300µl each of the three standard proteins (2mg/ml) so that the total amount of protein was approximately 1.8mg. Several drops of 2M sucrose were added to the protein mixture. The solution of standard proteins was carefully added above the gel as described earlier in the determination of the void volume. Prior to collecting individual fractions 24mls of PBS were eluted into a measuring cylinder. This procedure was carried out to reduce the number of unnecessary fractions collected, since the first protein to be eluted (aldolase) would be expected in the void volume. Two hundred 1ml fractions were collected in 13 x 100mm test tubes using an LKB 2070 ultrorac fraction collector. After elution, the concentration of protein was determined in all tubes by a spectrophotometer at a wavelength of 280nm. Three protein peaks were determined with the highest molecular weight standard eluted as the first peak. Although the absorbance (optical density) is expressed against fraction number, a known volume of PBS (24mls) was eluted prior to collection of fractions. Using these results and knowing the molecular weight of hCG it was possible to predict the fractions in which the FITC-hCG conjugate would be eluted.

Purification of FITC-hCG on a Sephadex G-75 column.

FITC-hCG conjugate was further purified on a Sephadex G-75 column. This procedure was necessary in order to remove other proteins from the solution since the hCG used for coupling to FITC was not pure grade. The pooled fraction containing the FITC-hCG conjugate eluted from the Sephadex G-25 column, together with several drops of 2M

sucrose solution was carefully transferred to the sephadex G-75 column as described previously. Based on the molecular weight of hCG and the results of the elution profile for the protein standards, 50mls of PBS was collected prior to elution of 1ml fractions. The concentrations of protein and fluorescein were determined for each fraction by a spectrophotometer at 280nm and 495nm, respectively. Tubes containing the highest concentration of protein and fluorescein were saved and stored at 4°C.

Determination of the biological activity of FITC-hCG.

To ascertain the fluorescein conjugated protein is hCG a radioligand receptor assay using rat ovarian homogenates was performed. The homogenates were prepared as described earlier. Apart from fractions determined as containing the highest concentrations of FITC and hCG, several fractions containing low concentrations of FITC and hCG were included in the assay to monitor the difference in nonspecific binding. From each fraction, 100µl of solution was added to one tube containing the homogenate. These tubes were used to determine the amount of FITC-hCG present in each fraction through measurement of the degree of competition with the ^{125}I-hCG. Fractions containing high concentrations of FITC-hCG would be expected to displace more ^{125}I-hCG from binding to receptors compared to fractions containing low concentrations of FITC-hCG. The amount of ^{125}I-hCG bound should thus be inversely related to the amount of FITC-hCG present. The remaining two tubes representing each fraction were used to measure total binding. Fifty microliters of ^{125}I-hCG (100,000cpm)

was added to all assay tubes and the total reaction volume of each tube corrected to 350µl with 0.1% BSA/PBS. After overnight incubation at room temperature the reaction was stopped, with 3ml of 0.1 BSA/PBS. The reaction mixture was centrifuged at 3000 rpm for 30 minutes to remove unbound hormone. The supernatant was aspirated and the pellet counted for radioactivity in a gamma counter (Beckman 4000). Fractions were selected for in vitro studies on the basis of the lowest amount of specific binding measured and the highest absorbance levels obtained at both 280nm and 495 nm. The conjugate was stored in 50µl aliquots in microcentrifuge tubes at -20°C.

b. Coupling of tetramethylrhodamine isothiocyanate (TRITC) with ovine prolactin (oPRL) and purification of conjugate. The procedures for the conjugation of TRITC and oPRL and the subsequent purification of the conjugate were identical to that of FITC-hCG. TRITC (Research Organics Inc., Cleveland Ohio; 0.25mg) was coupled to 5mg OPRL (31IU/mg) using the procedure described earlier in section (a) for FITC-hCG except no radioactive hormone was added to monitor protein recovery. The reaction mixture was concentrated under vacuum overnight and dialysed against PBS as previously described.

Column chromatography (Sephadex G-25; 10ml column) was used to separate TRITC-oPRL from free TRITC. One milliliter fractions were eluted from the column with PBS and the concentration of protein and fluorochrome determined by a spectrophotometer at 280nm and 560nm, respectively. Fractions containing the highest concentration of TRITC

and oPRL were pooled and concentrated under vacuum to a volume of approximately 1ml and then dialysed against PBS.

Further purification of the TRITC-oPRL conjugate was carried out as described earlier for FITC-hCG. Tubes containing the highest concentration of oPRL and TRITC were retained. Aliquots (200µl) from these fractions were pipetted into microcentrifuge tubes, covered with foil and stored at -20°C. Assessment of the biological activity of this conjugate was not performed.

2.3. Localization of LH and PRL Receptors in Cultures of Rat Luteal Cells Using FITC Conjugated to hCG and TRITC Conjugated to oPRL

Granulosa-lutein cell cultures from PMSG primed rats were established as described earlier in the Materials and Methods section of this chapter (Section 2.1, part (a)). Conjugated fluorochromes (50µl) were added to selected cultures (days 1-4 after plating) either alone or in combination and incubated at 37°C for either 15 minutes or 2 hours. After incubation the coverslip containing attached cells was removed from the petri dish and washed in PBS to remove unbound fluorochrome conjugated hormones. The coverslip was turned face-down on a drop of media (Eagle's-Ham's) located on a slide and the edges sealed with wax. The slide was rinsed with distilled water to remove excess PBS. Cells were viewed using a Nikon Labophot microscope with fluorescence. For observation of rhodamine, a K-610 filter was used. Photographs were taken using a Nikon 35mm camera attached to the microscope. Single and double exposures were taken with the length of exposure varying from 2-5 minutes. To determine the amount

of nonspecific binding of the fluorochromes, cells were incubated with an excess of either unlabelled hCG or oPRL prior to the addition of fluorescently labelled hormones.

3. Results

3.1. Elution of Standard Proteins on a Sephadex G-75 Column

To determine the fraction expected to contain hCG and oPRL with their respective conjugated fluorochromes a series of standard proteins was applied to a sephadex G-75 column and eluted with PBS. The concentration of protein in each fraction was determined by spectrophotometric absorbance and the results shown in Figure 12. Three protein peaks were obtained with the highest molecular weight protein, aldolase, eluted in the first peak. Lower molecular weight proteins, ovalbumin and chymotrypsinogen A were eluted in the second and third peaks, respectively. The fraction containing the highest concentration of aldolase was designated as the void volume (v_o). Theoretically, since the molecular weights of both aldolase and blue dextran (B.D.) are high, both molecules should be eluted in the same fractions and the v_o defined as those fractions which contain both aldolase and blue dextran. From these results it was possible to determine from the fractions eluted those expected to contain FITC-hCG and TRITC-oPRL.

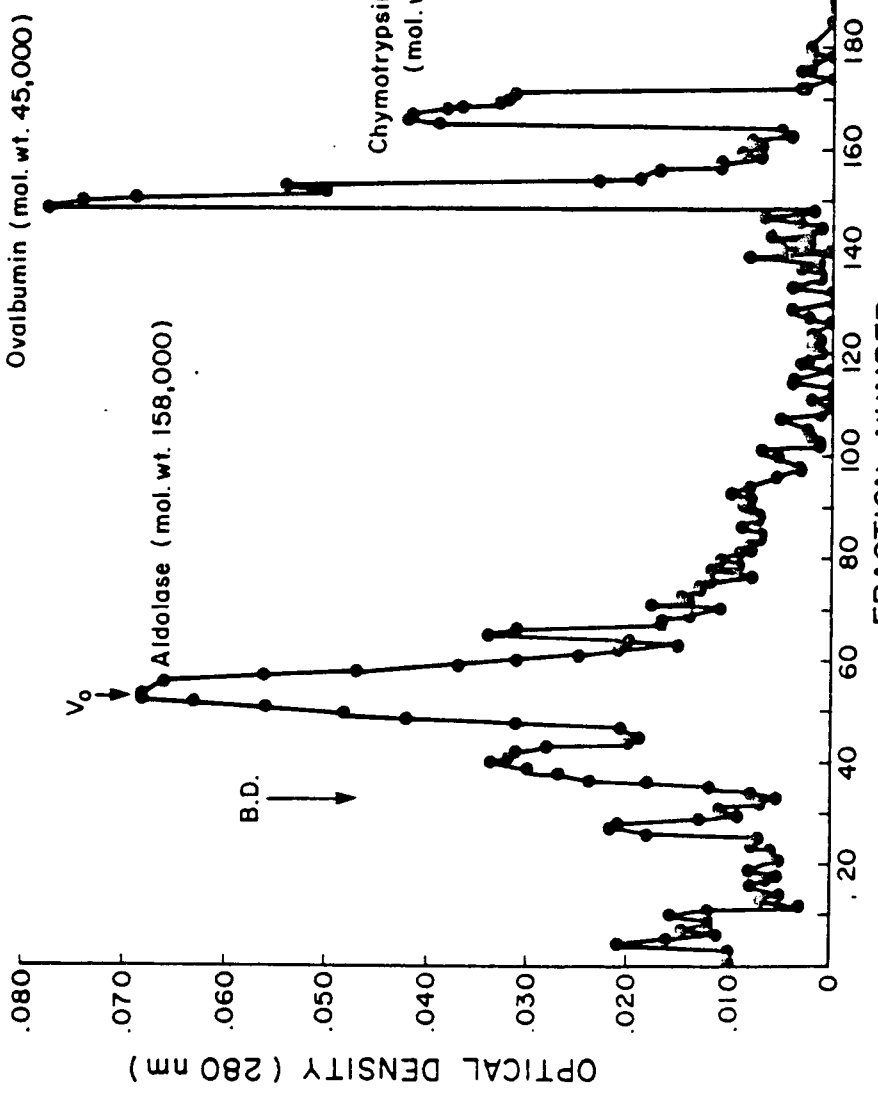

Figure 12. The elution profile for standard proteins from a Sephadex G-75 column. Each fraction represents 1ml eluted, with 24mls of PBS collected prior to elution. Blue dextran (BD); void volume (v_o).

3.2. Purification of FITC-hCG and TRITC-oPRL on a Sephadex G-75 Column

Fractions containing fluorochrome conjugates, initially purified on a sephadex G-25 column, were selected for further purification on a sephadex G-75 column. Selection of fractions 5-7 containing FITC-hCG and fractions 6-8 containing TRITC-oPRL was based on the highest concentrations of protein and fluorochrome present in each fraction. Figure 13 illustrates the elution profile of FITC-hCG from a Sephadex G-75 column. Fractions 1-8 contained the highest concentrations of hCG and FITC. These fractions were used to localize LH receptors in rat luteal cells *in vitro*.

The elution profile of TRITC-oPRL from a Sephadex G-75 column is shown in Figure 14. Although a small protein-fluorochrome peak was observed at the beginning of collection the major peak corresponded to fraction number 27. This fraction was used to localize PRL receptors in cultured rat granulosa cells.

3.3. Binding Activity of FITC-hCG to Ovarian Homogenates

To confirm FITC conjugated protein contains biological activity a radioreceptor assay was carried out. The degree of competition was determined using 100µl of the solution from each fraction. The results are shown in Table 1 and are expressed as cpms of specifically bound ^{125}I-hCG. Low specific binding in fractions 1-27 confirmed the presence of high levels of hCG in these fractions and demonstrates the biological activity of the conjugate in these fractions.

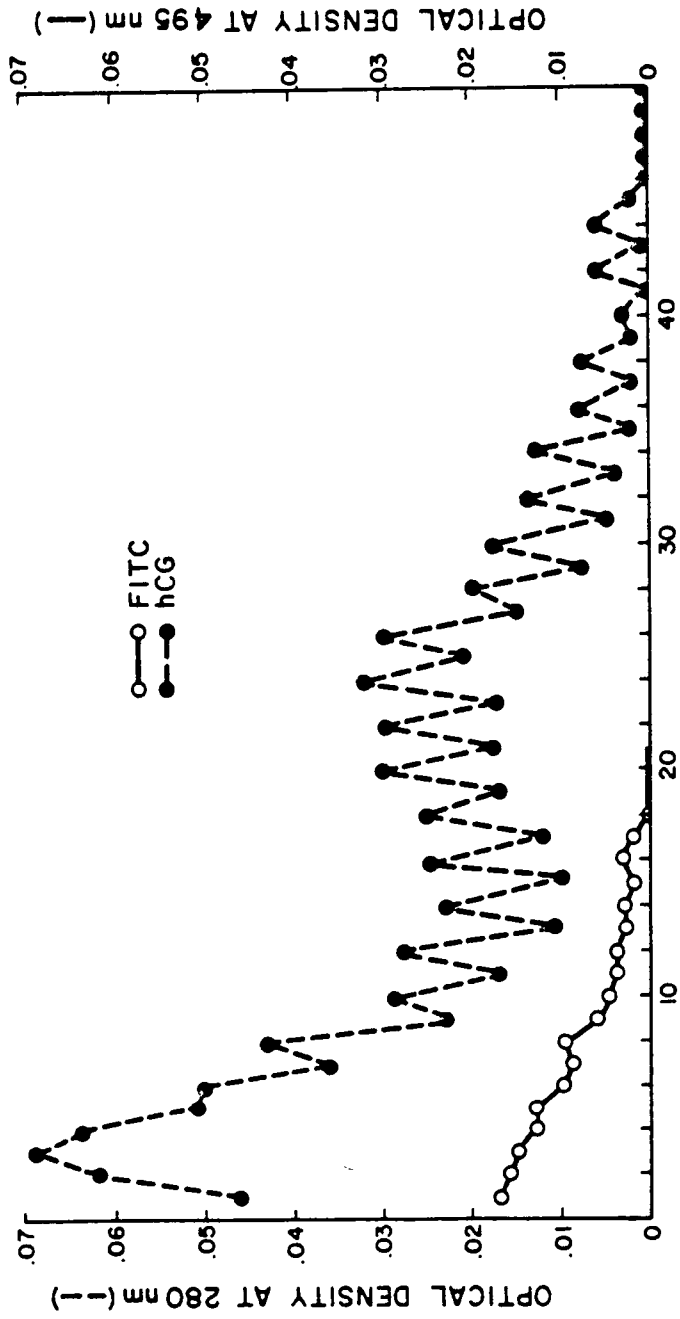

Figure 13. The elution profile of FITC-hCG from a Sephadex G-75 column. Fifty milliliters of PBS was eluted prior to collection of 1ml fractions.

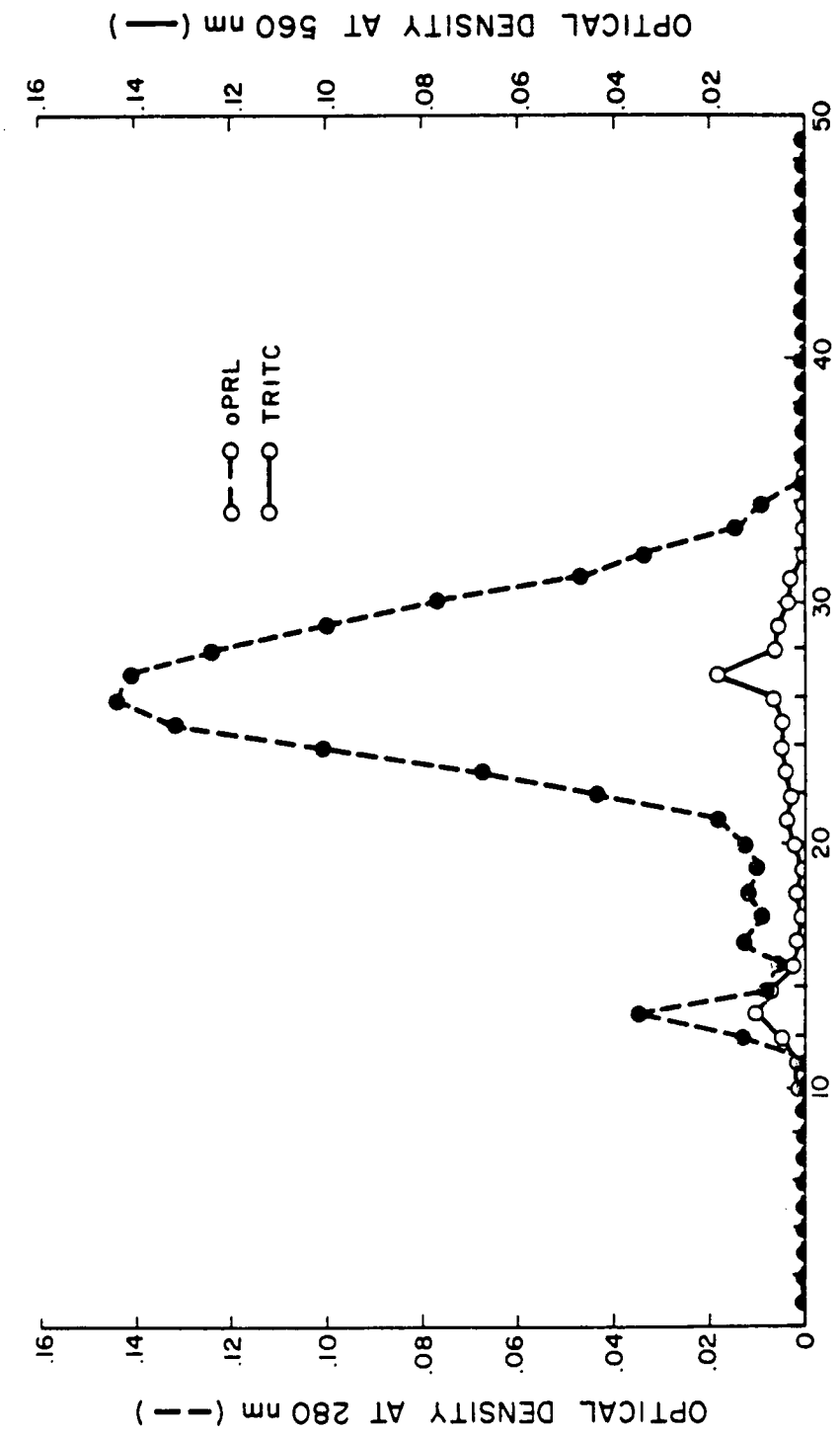

Figure 14. The elution profile of TRITC-oPRL from a Sephadex G-75 column. Sixteen milliliters of PBS was eluted prior to collection of 2ml fractions.

Table 1. Specific binding of ^{125}I-hCG to ovarian homogenates

Fraction No.	Specific Binding (cpm)	Fraction No.	Specific Binding (cpm)	Fraction No.	Specific Binding (cpm)	Fraction No.	Specific Binding (cpm)
1	<500	11	608	21	1398	31	19961
2	<500	12	992	22	1883	32	23292
3	725	13	642	23	2390	33	22229
4	<500	14	720	24	5965	34	22080
5	<500	15	948	25	2438	35	21487
6	<500	16	1538	26	4839	36	22724
7	<500	17	642	27	4737	37	22582
8	<500	18	903	28	10161	38	24256
9	<500	19	1330	29	13972	39	22560
10	<500	20	1215	30	17318	40	22393

Note: Fractions containing hCG conjugated to FITC were obtained by chromatography on a Sephadex G-75 column, and were used to determine the degree of competition with ^{125}I-hCG in a radioreceptor assay. Specific binding of ^{125}I-hCG was reduced with high concentrations of FITC-hCG. Total binding was approximately 22,000cpm.

3.4. The Effect of hCG on LH Receptors in Cultured Rat Granulosa-Lutein cells

Cell cultures were photographed at 200x (Plate VIII) prior to and after the addition of hCG. Changes in the number of lipid droplets, the appearance of vacuoles at the time of regression and overall growth of the cells is recorded in the legend for Plate VIII.

Prior to localization of LH and PRL receptors in cultured rat luteal cells through their respective fluorochrome conjugates it was necessary to determine whether a desensitizing dose of hCG would result in a decrease in the number of LH receptors. In this experiment the effect of a high concentration of hCG on ^{125}I-hCG binding to luteal cells *in vitro* was not clearly demonstrated. At days 1 to 4 inclusive (day 0 = day of hCG treatment) minimal specific binding was observed in both the control and hCG treated groups. However, subtle changes in receptor numbers may reflect a desensitizating effect of hCG on these cells, particularly since at day 1 no specific binding was measured in cells treated with hCG while moderate binding (~1000cpm) was measured in the control group. Significant radioactivity was not observed until day 4 in the treatment group while in the control group specific binding, although not measurable at day 2, increased from day 3.

3.5. The Effect of hCG on Steroid Production by Cultured Rat Granulosa-Lutein Cells

The effect of a high dose of hCG on progesterone production was determined to emphasize not only a desensitizing role in terms of reduced progesterone levels but also to correlate a possible association

Plate VIII. Light photomicrographs of cultured rat granulosa-lutein cells at various times after plating.

Figure A. Granulosa-lutein cells at day 1 after plating. Cells appear small and rounded with few lipid droplets (x200).

Figure B. Granulosa-lutein cells at day 3 after plating. Cells have large extensive processes and contain many lipid droplets (x200).

Figure C. Granulosa-lutein cells at day 4 after plating. Cells have spread out considerably and several show early signs of vacuolization (x200).

Figure D. Granulosa-lutein cells at day 6 after plating. Many cells that are regressing show extensive vacuolization. Lipid content has decreased (x200).

Figure E. Granulosa-lutein cells at day 7 after plating. Several cells show extensive vacuolization. Cells contain very little lipid (x200).

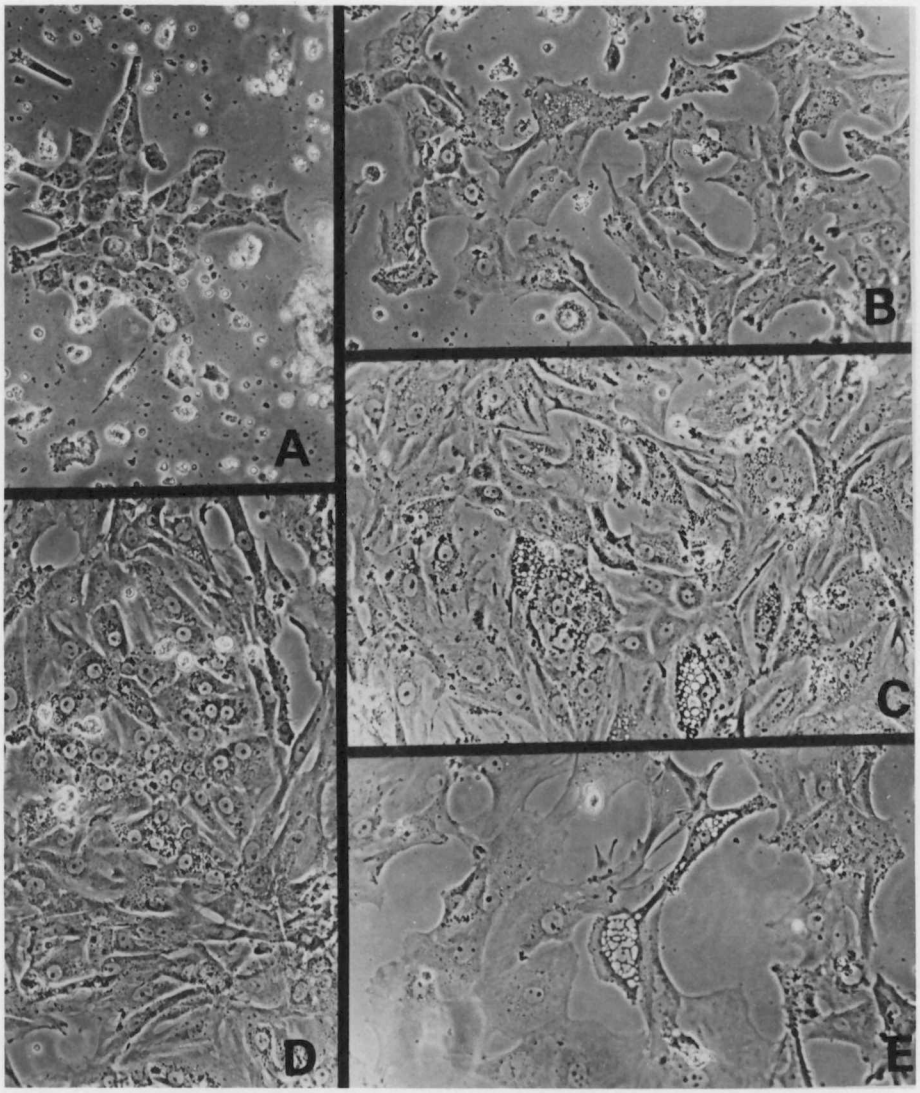

Plate VIII

between changes in progesterone output and the number of LH receptors. High concentrations of progesterone (200-250ng/dish/24 hours) were measured in the media which was obtained prior to the addition of hCG to cell cultures. Furthermore, the concentration of progesterone was maintained at these high levels in both control and treatment groups with statistically no significant difference between either group over the time points examined.

Estrogen levels in the media were also determined from both control and hCG treated groups and were initially found to be less than 2ng/dish/24 hours at day 2 after plating. Levels dropped rapidly over the next five days to undetermined values. Results of both the progesterone and estrogen assays indicate that the cells were fully luteinized.

3.6. Localization of LH and PRL Binding Sites in Cultured Granulosa Lutein Cells

To localize receptors for both LH and PRL in rat luteal cells, FITC-hCG and TRITC-oPRL were added to cultures of granulosa-lutein cells and allowed to incubate for periods of either 15 minutes or 2 hours at 37°C. In Plate IX, figure A, the cell was exposed to FITC-hCG during incubation for a period of 15 minutes. The fluorochrome is sparsely concentrated around the periphery of the cell. Individual fluorescence observed distal to the circular arrangement of the fluorescein represents localization of the fluorochrome to spindle like processes of the cell. In figures B and C the fluorescein is concentrated around the nuclei of two

Plate IX. Localization of FITC-hCG or TRITC-oPRL in cultured rat granulosa-lutein cells.

Figure A. Incubation of FITC-hCG with cultured cells for 15 minutes. A single cell showing localization of FITC-hCG around the periphery of the cell.

Figures B and C. Incubation of FITC-hCG with cultured cells for 2 hours. Fluorescent staining is concentrated primarily around the nuclei.

Figure D. Incubation of TRITC-oPRL with cultured cells for 2 hours. The fluorescence is localized around the nuclei (arrow) of two cells.

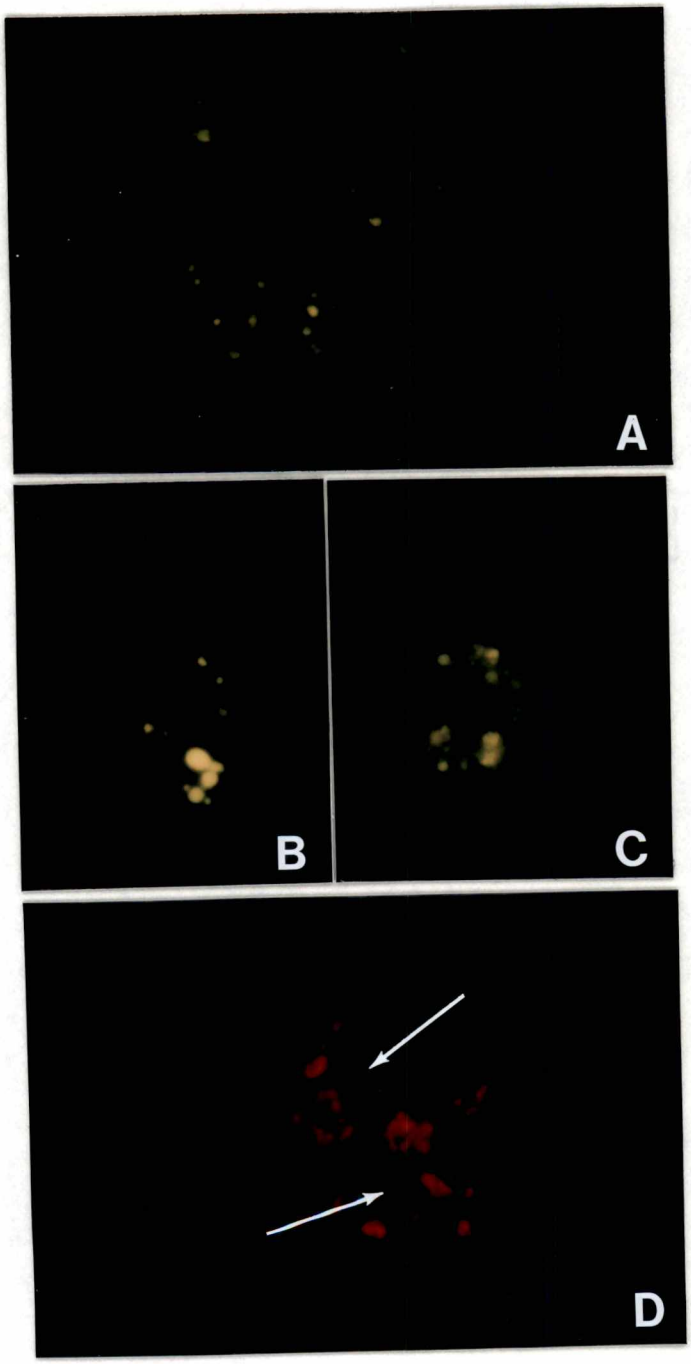

Plate IX

single cells which were exposed to the conjugate during incubation for approximately 2 hours. In figure D rhodamine appears to concentrate heavily around the nuclei of two cells which were incubated with TRITC-oPRL for approximately 2 hours.

In Plate X, figures A-C represent a double exposure of fluorescein and rhodamine. In figure A the cell was incubated with both FITC-hCG and TRITC-oPRL for 15 minutes while in figures B and C the cells were exposed to the fluorochrome conjugates for 2 hours. In figure A both FITC-hCG and TRITC-oPRL appear to be closely associated with each other around the periphery of the cell. Figure B shows the pattern of distribution of the fluorochrome conjugates close to the nucleus of a single cell while in figure C the fluorescence is in the perinuclear region of two adjacent cells.

In all figures distinct areas of localization of both fluorochromes are shown interspersed with areas exhibiting more diffuse staining. Nonspecific staining was low in cells treated with unlabelled hCG or oPRL prior to incubation with fluorescently labelled hCG or oPRL.

4. Discussion

The biological response of a cell is dependent upon the binding of a particular ligand to specific receptors located on the plasma membrane (Catt and Dufau, 1976; 1977). Localization of receptors on the surface of cells has been demonstrated using several methods including radiolabelling and fluorescent labelling techniques

Plate X. Simultaneous localization of FITC-hCG and TRITC-oPRL in cultured rat granulosa-lutein cells.

Figure A. Incubation of FITC-hCG and TRITC-oPRL with cultured cells for 15 minutes. Fluorescence from both FITC-hCG and TRITC-oPRL are localized together in small patches around the periphery of the cell.

Figures B and C. Incubation of FITC-hCG and TRITC-oPRL with cultured cells for 2 hours. Fluorescence from both FITC-hCG and TRITC-oPRL is localized together in the center of the cells. In figure B the pattern of fluorescent staining is localized within a single cell while in figure C the pattern is localized within two cells (arrow) that are close together.

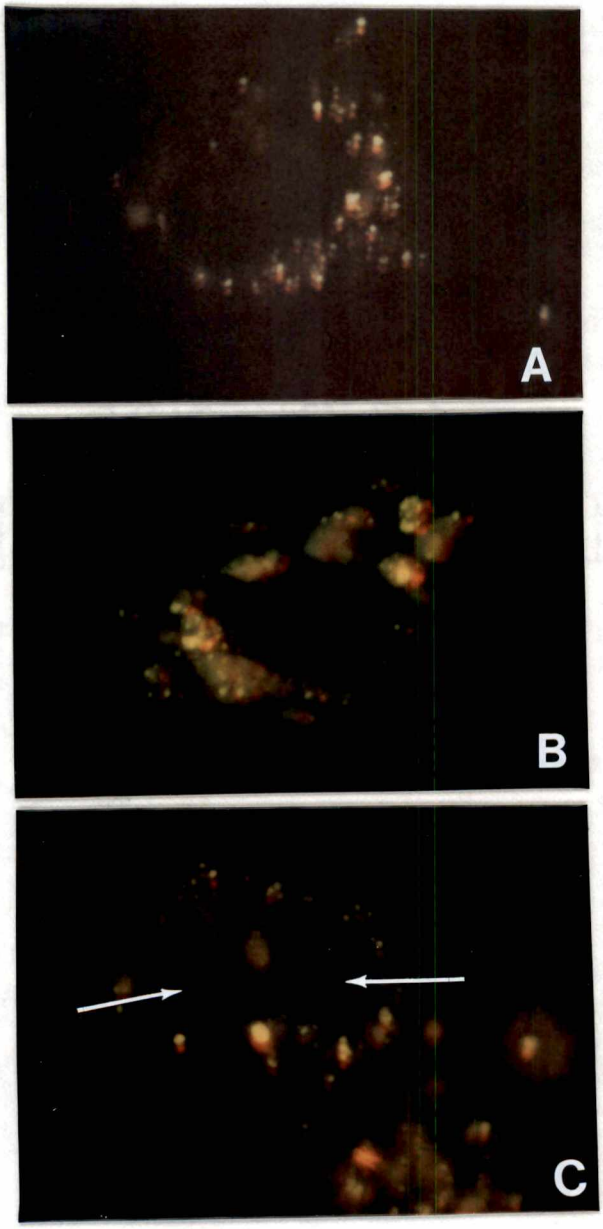

Plate X

(Ahmed et al., 1981; Pasten and Willingham, 1981). These techniques have also been employed to demonstrate internalization of the receptor-hormone complex followed by degradation of the hormone.

In the present series of experiments the temporal relationship between LH and PRL receptors during desensitization was further investigated by adding purified fluorochrome-hormone conjugates to cultures of rat granulosa-lutein cells. Both fluorochrome conjugates were purified by column chromatography to remove traces of unbound fluorochrome and extraneous protein. The resulting preparations were considered to be reasonably pure in view of their elution profiles. Only those fractions from the major peak of each elution profile, that contained the highest concentration of protein and fluorochrome, were selected. In addition to being acceptable as a marker for either hCG/LH or PRL the fluorochrome conjugate should possess significant biological potency. This was determined only for the FITC-hCG conjugate using a radioreceptor assay and the results demonstrated significant biological activity in fractions containing high concentrations of FITC-hCG.

The pattern of distribution of the fluorochromes in cultures incubated for 15 minutes with either FITC-hCG or TRITC-oPRL, alone, or in combination, was mainly around the periphery of the cell. However, with longer periods of incubation the fluorochromes became distributed around the perinuclear region. At both short term and long term incubations the fluorescence is seen as visible patches rather than as diffuse staining. It therefore would be reasonable to assume that, during the initial stages of incubation, hCG and PRL

bind to their receptors on the plasma membrane which then initiates clustering into patches. These clusters of receptors are then internalized in vesicles which then concentrate around the nucleus possibly in the Golgi region. Avivi et al. (1982), using rhodamine conjugated to thyroid stimulating hormone (TSH) also demonstrated a similar pattern of binding and internalization of the hormone-receptor complex. In their experiments rhodamine was diffusely distributed over the surface of the cells at 4°C, while at 37°C patches were rapidly formed within 15 minutes with the rhodamine observed in endocytic vesicles within the cells.

Interestingly, when cells were incubated with both FITC-hCG and TRITC-oPRL either for 15 minutes or 2 hours the fluorochrome conjugates were found to be closely associated with each other. The patches of fluorescein and rhodamine appear quite distinct from each other. However, some areas do not appear clearly defined and this may be attributed to vibrations occurring at the time of photographic exposure. Since the time of exposure was very lengthy (up to 5 minutes), minute vibrations at the time may cause a slight shifting in the exposure of one of the fluorochromes. Nevertheless, the fact that both FITC and TRITC are found close together in localized areas suggests that LH and PRL receptors are closely associated with each other. Finally, the apparent diffuse staining of FITC around the nucleus of several cells may be due to lysosomal breakdown of the endocytic vesicles containing the FITC-hCG conjugate with a subsequent release of fluorochromes.

These studies point to the possibility that in the rat corpus luteum, receptors for the luteotropic hormones PRL and LH are closely associated with each other. Since both hormones are required for the continued maintenance of progesterone production it would be reasonable to expect that during periods of desensitization, due to high levels of circulating LH (or PRL), receptors for both LH and PRL are down regulated within the same vesicle. This phenomenon would be of considerable advantage to target tissue as it would reduce the sensitivity of the cells to continued stimulation. Cointernalization of two or more receptors within the same vesicle has also been shown by Maxfield et al. (1979) using fluorescein conjugated to $\alpha_2$macroglobulin and rhodamine conjugated to insulin and epidermal growth factor (EGF). The mechanism of internalization of LH and PRL receptors may be similar to that proposed for $\alpha_2$macroglobulin and EGF, in that binding of the hormone to its receptor stimulates clustering of receptors into coated pits. These hormone-receptor complexes, together with unoccupied heterologous receptors, are endocytosed within coated vesicles. It is possible that high levels of circulating LH/hCG bind to LH receptors and initiate lateral movements of both homologous and heterologous receptors. This results in cointernalization of both PRL and LH receptors within the same endocytotic vesicle. Clustering may occur in clathrin coated areas as proposed for the LDL, EGF and $\alpha_2$macroglobulin receptors (Maxfield et al., 1979). It also is important to consider that cointernalization of multiple receptors may simply represent a generalized effect on cell membrane receptor

turnover. However, Davies et al. (1980) showed that in the luteinized rat ovary there was no change in receptors for insulin and β-adrenergic ligands after a desensitizing dose of hCG even though receptors for LH and PRL decreased.

Finally, although radioreceptor studies and fluorescent staining techniques reveal a definite association between LH and PRL receptors in the rat corpus luteum, an element of independence does exist since the recovery of both receptors after down regulation occurs at different rates. This may be the result of different rates of receptor synthesis and/or recycling. Recycling of receptors may involve direct passage to the cell membrane or processing in the Golgi prior to transport to the cell membrane. The possibility that recovery of receptors involves the formation of new corpora lutea should, however, not be overlooked.

The results of these studies indicate a close association between the luteotropic receptors, LH and PRL, in the rat corpus luteum. This relationship is emphasized during down regulation of both receptors and is of considerable importance as a mechanism to reduce overstimulation of the target cell.

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APPENDIXES

APPENDIX A

MATERIALS

Tissue Fixation and Staining

1. Glutaraldehyde (4%): 80ml, 25% glutaraldehyde (Polysciences) and 170ml of distilled water was added to a 250ml solution containing 0.2M sodium cacodylate buffer (Sigma) and 10% sucrose; pH was adjusted to 7.4.
2. Osmium tetroxide (1%): a 4% solution of osmium tetroxide first prepared using crystal from one vial of osmium tetroxide (Polysciences) and dissolving in 25ml of distilled water. Five milliliters of the 4% solution was then diluted with 5ml of distilled water and 10ml of 0.2M sodium cacodylate buffer to make a 1% osmium tetroxide solution in 0.1M sodium cacodylate buffer.
3. Uranyl acetate solution: Two grams of solid was dissolved in 100ml of 70% ethanol. Saturatuion of the solution was obtained by adding a small amount of uranyl acetate crystals to the solution.
4. Lead citrate solution: 0.2mg of lead citrate was dissolved in a vial containing 10ml of distilled water and 100µl of 10N NaOH.
5. Propylene oxide and embedding media (Polybed 12) came from Polysciences.

Radioiodination

1. Phosphate solution (0.1M): 70ml of 0.1M Na_2HPO_4 (1.42g/100ml) was added to 30ml of 0.1M NaH_2PO_4 (1.38g/100ml) and the pH adjusted to 7.1. The solution was aliquotted into 500µl samples and stored at -20°C.
2. Hydrogen peroxide solution: 20µl of 1:400 stock solution of hydrogen peroxide was added to 4ml of distilled water in a non-glass vial to obtain a 1.80,000 dilution. The 1:400 stock solution was made by adding 500µl of 30% hydrogen peroxide solution (Sigma) to 200ml of distilled water. To check the solution, 250µl of the 30% solution was added to 100mls of distilled water and the dilution checked using a U.V. spectrophotometer set at a wavelength of 230nm (Absorbance = 1.6).
3. Rinse and Transfer solutions: these were prepared as 8% and 16% sucrose solutions, respectively, in 0.1% BSA.PBS and stored in 500µl aliquots at -20°C.
4. Sephadex G-25: the beads were prepared the day before radio-iodination by dissolving a small quantity of sephadex G-25 (Sigma) in distilled water and swollen at 4°C.

Steroid Assay

1. Charcoal: 0.5g T-70 dextran (Pharmacia, Uppsala, Sweden) and 5g Norit A charcoal (Biochemical Corporation, Cleveland, Ohio) was mixed thoroughly on ice in 200ml 0.1% BSA/PBS to obtain a 10X

solution. For the assay a 1X solution was obtained by diluting according with 0.1% BSA/PBS.

2. Scintillation Fluids: 16g of PPO (2,5-Diphenyloxazole) and 0.32g of bis-MSB (p-Bis-(o-Methylstyryl)Benzene) was added to 4 liters of toluene. PPO and bis-MSB are scintanalyzed fluors obtained from Fisher Scientific Co., Fair Lawn, New Jersey.

Other Chemicals

1. Dulbecco's PBS: the following chemicals were used to prepare a 1X solution of phosphate buffered saline (pH 7.4); KCl-0.2g/L, NaCl-8.0g/L, Sodium Azide -1.0g/L, KH_2PO_4-0.2g/L, Na_2HPO_4-1.14g/L.
2. 0.1% BSA/PBS: 0.4g BSA (Bovine Serum Albumin from Sigma) was dissolved in 400ml of PBS.
3. Eagle's-Ham's media: minimum essential medium (Eagle) (MEM) was obtained from Gibco Laboratory, Grand Island, New York. 2.2g of $NaHCO_3$ and 2.38g of Hepes was added to 1 liter of distilled water containing a packet of MEM. The pH was adjusted to 7.4 and the solution filter sterilized through a 0.45μm millipore filter. Ham's F12 (modified) media was purchased from Flow Laboratories in Virginia. 10.64g of Ham's medium and 5.95g of Hepes was added to 1 liter of distilled water and the pH adjusted to 7.4. The solution was filter sterilized and added to prepared Eagle's media. The final pH was brought to 7.4. Fungizone (0.2%) and 2% penicillin and streptomycin were added to the media.

APPENDIX B

DETERMINATION OF PROTEIN CONTENT OF OVARIAN HOMOGENATES

The Bradford assay was used to determine the amount of membrane protein used in the binding studies. Fifty microliters of ovarian homogenate (one ovary/2ml PBS) was pipetted in triplicate into test tubes containing 50µl of 1N NaOH and allowed to incubate for 10 minutes. The total volume was brought up to 150µl with PBS. Standard protein concentrations (10-50µg 0.1% BSA/PBS) were established in triplicate and corrected for a total volume of 50µl with PBS. Bradford reagant (2.5ml) was added to all tubes and protein concentrations determined using a Perkin Elmer Lambda 3 uv/vis spectrophotometer at a wavelength of 595nm. A regression line of best fit was determined using the data obtained from the standard protein concentrations. The amount of protein in the homogenate was determined using the mean absorbance readings from the sample tubes. In all future radioreceptor assays 100µl of membrane homogenate containing approximately 100µg protein was used.

The Bradford reagant was prepared by dissolving 100mg of Coomassite Brilliant Blue G-2 in 50ml of 95% ethanol and 100ml of 85% phosphoric acid. The solution was brought up to one liter with distilled water.

APPENDIX C

BINDING OF ^{125}I-hCG TO DIFFERENT DILUTIONS OF MEMBRANE HOMOGENATE

To determine the desired concentration of ovarian homogenate required to bind a saturable quantity of radiolabelled hCG, several dilutions (1:5, 1:10, 1:15, 1:20) of homogenate were prepared from a stock homogenate (one ovary/2ml PBS). In the presence or absence of excess unlabelled hCG, binding of ^{125}I-hCG (~125,000cpm) was determined for each membrane homogenate. After overnight incubation at room temperature 3ml of 0.1% BSA/PBS was added to all tubes. Separation of unbound labelled hormone from bound hormone was carried out as described in the Materials and Methods from Chapter II. The pellet was counted for radioactivity and specific binding calculated as the difference between totals and nonspecific binding. The results are shown in Table C-1 and are expressed as specific binding in cpm and as a percentage of the total radioactivity added to the tubes. For future binding studies involving hCG, a 1:5 dilution of the homogenate was used.

Table C-1. Binding of ^{125}I-hCG to various dilutions of membrane homogenate in 50 and 100µl volume samples.

Volume of Membrane Homogenate	Dilution of Membrane Homogenate			
	1:5	1:10	1:15	1:20
50µl	23649	8277	4647	3153
	23.6%	6.6%	3.7%	2.5%
100µl	26643	12440	6941	6689
	26.6%	10.0%	5.6%	5.4%

Note: Results are expressed as specific binding in cpm (upper) and as percent of total radioactivity added (lower). Specific binding was determined as a mean of three replicates.

APPENDIX D

COMPARISON OF THE EFFECT OF USING PBS OR 0.1% BSA/PBS IN REDUCING NONSPECIFIC BINDING IN RADIORECEPTOR ASSAYS

To determine the effect of PBS and 0.1% BSA/PBS in reducing nonspecific binding in radioreceptor binding studies, ovarian membrane homogenates (1:5 dilution) were added in 50 and 100µl aliquots to tubes containing labelled hCG (~100,000cpm/50µl) with or without an excess (10µg) of unlabelled hCG. To the tubes containing unlabelled hormone 240µl of either PBS or 0.1% BSA/PBS was added while in tubes without unlabelled hCG 250µl of either buffer was added. The remainder of the procedure to determine the amount of specific binding of labelled hCG is described in the Materials and Methods from Chapter II. The results are shown in Table D-1 and illustrate the effect of PBS and 0.1% BSA/PBS on nonspecific binding and the determination of specific binding. Since 0.1% BSA/PBS was more effective in reducing nonspecific binding compared to PBS it was used in all subsequent receptor binding studies.

Table D-1. The effect of PBS and 0.1% BSA/PBS on nonspecific (NS) and specific (S) binding of ^{125}I-hCG in 50 and 100µl volume samples of ovarian membrane homogenate. Results are expressed in cpm.

Volume of Homogenate	PBS		0.1% BSA/PBS	
	NS	S	NS	S
50µl	2297	11805	1635	12244
100µl	2260	12649	1737	13752

APPENDIX E

Table E-1. LH and PRL receptors in corpora lutea of superovulated rats at 6, 24 and 48 hours after treatment with different doses of $PGF_{2\alpha}$

Dose of $PGF_{2\alpha}$	LH Receptors (cpm x 10^3/ovary)				PRL Receptors (fmoles/ovary)			
	0h	6h	24h	48h	0h	6h	24h	48h
Control	1137.3 ±109.1	1317.8 ±25.6	1234.5 ±80.4	1280.7 ±28.2	271.5 ±73.2	351.1 ±30.3	241.2 ±82.8	167.1 ±26.9
0.22mg	*	1134.1 ±71.2	1164.3 ±196.7	1344.6 ±137.3	*	196.6 ±27.5	252.8 ±87.9	289.4 ±47.0
0.66mg	*	1163.5 ±101.7	1235.7 ±78.1	1271.4 ±131.0	*	154.3 ±35.8	239.7 ±26.7	269.9 ±67.6
2.0mg	*	1166.1 ±91.0	999.7 ±69.9	1297.8 ±135.9	*	99.7 ±16.6	157.9 ±49.5	276.8 ±29.6

Results are expressed as mean ± standard error of the mean (n=4).

*Treated animals not sacrificed at 0 hours.

Table E-2. Experiment I: LH receptors in corpora lutea of superovulated rats determined at five hourly intervals for up to 25 hours after treatment with hCG, GnRH and $PGF_{2\alpha}$

Treatment	LH Receptors (cpm x 10^3/ovary)					
	0h	5h	10h	15h	20h	25h
Control	1805.7 ±220.6	1795.5 ±610.2	1564.2 ±305.6	1722.7 ±17.3	1805.6 ±106.4	1383.2 ±187.7
hCG	*	1769.7 ±172.9	991.0 ±167.0	1039.4 ±361.3	594.9 ±533.1	470.6 ±27.0
GnRH	*	1416.7 ±116.4	1249.8 ±355.9	1157.1 ±212.9	709.8 ±70.6	610.0 ±378.5
$PGF_{2\alpha}$	*	1147.8 ±141.3	989.7 ¼729.5	1370.2 ±74.7	925.2 ±207.9	**

Results are expressed as mean ± standard error of the mean (n=3).

*Treated animals not sacrificed at 0 hours.

**Not computed.

Table E-3. Experiment II: LH and PRL receptors in corpora lutea of superovulated rats determined at one day intervals, for up to five days, after treatment with hCG, GnRH and $PGF_{2\alpha}$

Treatment	LH Receptors (cpm x 10^3/ovary)						PRL Receptor (fmole/ovary)					
	0d	1d	2d	3d	4d	5d	0d	1d	2d	3d	4d	5d
Control	2249.1 ±276.9	3337.1 ±124.9	2931.1 ±346.5	2350.1 ±581.1	1769.0 ±1372.1	3133.5 ±17.4	332.9 ±55.7	324.9 ±22.8	321.4 ±58.3	289.9 ±31.5	258.5 ±8.3	311.2 **
hCG	*	709.5 ±276.9	487.0 ±868.0	2514.8 ±287.2	2767.7 ±388.8	3387.8 ±472.5	*	255.9 ±55.4	356.9 ±6.7	352.9 ±29.0	284.9 ±60.9	317.0 ±0.5
GnRH	*	1914.9 ±473.2	1646.1 ±840.6	2062.1 ±562.8	2795.1 ±297.7	772.2 ±459.3	*	289.9 ±29.1	330.8 ±49.3	296.1 ±22.8	373.5 ±51.9	161.4 ±47.7
$PGF_{2\alpha}$	*	2305.5 ±117.3	2405.9 ±197.2	2972.6 ±256.2	3317.2 ±182.0	1946.2 ±900.8	*	227.0 ±22.3	259.3 ±13.5	312.1 ±82.6	290.4 ±27.2	204.2 ±61.6

Results are expressed as mean ± standard error of the mean (n=3).

*Treated animals not sacrificed at time 0.

**One animal only.

Table E-4 Experiment III: LH and PRL receptors in corpora lutea of superovulated rats determined at two day intervals, for up to eight days, after treatment with hCG, GnRH and $PGF_{2\alpha}$

Treatment	LH Receptors (cpm x ^{3}10/ovary)						PRL Receptors (fmoles/ovary)					
	0h	6h	2d	4d	6d	8d	0h	6h	2d	4d	6d	8d
Control	2328.7 ±403.0	2952.0 ±178.9	2871.1 ±151.0	2701.6 ±182.3	2596.0 ±510.8	2201.7 ±686.8	155.0 ±37.7	296.2 ±82.4	275.4 ±46.7	225.7 ±42.5	272.0 ±52.0	297.9 ±94.7
hCG	*	2250.0 ±440.5	656.4 ±225.2	2692.5 ±257.8	2160.2 ±1144.7	1863.5 ±960.1	*	155.6 ±37.9	382.0 ±115.0	302.3 ±59.3	219.6 ±50.3	341.1 ±32.1
GnRH	*	2739.2 ±49.2	2289.1 ±225.5	2565.3 ±213.9	2614.6 ±195.6	818.6 ±839.6	*	138.6 ±29.6	309.6 ±69.7	273.9 ±71.5	201.6 ±54.9	207.1 ±29.9
$PGF_{2\alpha}$	*	2225.5 ±47.5	2153.0 ±678.3	2201.9 ±443.9	1172.9 ±980.0	323.0 ±183.3	*	55.1 ±14.5	277.2 ±53.6	368.5 ±80.9	283.6 **	262.3 ±39.4

Results are expressed as mean ± standard error of the mean (n=5 for control; n=4 for treatments).

*Treated animals not sacrificed at time 0.

**One animal only.

APPENDIX F

Table F-1. The effect of 4M $MgCl_2$ on specific binding of ^{125}I-hGH using different volumes of membrane homogenate

Amount of Membrane Homogenate (µl)	Before $MgCl_2$		After $MgCl_2$	
	Specific Binding (cpm)	Percent of Total Radioactivity Added	Specific Binding (cpm)	Percent of Total Radioactivity Added
50	338	<1	33347	41
100	2018	24	32748	40
150	36	<1	36182	45
200	1058	13	33902	42

Results are expressed as cpm of specifically bound radiolabelled hormone and as percent of total radioactivity added.

APPENDIX G

DILUTION OF PROGESTERONE ANTIBODY TO ACHIEVE 50% BINDABILITY

To determine the concentration of progesterone antibody required to achieve a buffer control value of 50% bindability, 100µl of stock antibody (1:10 dilution) was diluted with 0.1% BSA/PBS. Dilutions ranged from 1:100 to 1:1000. Fifty microliters of each antibody concentration was added in triplicate to tubes containing 50µl of tritiated progesterone and 250µl of 0.1% BSA/PBS. Additional tubes, representing total and nonspecific binding, contained radiolabelled progesterone and 0.1% BSA/PBS only. Incubation was carried out for 17 hours at 4°C. Separation of bound ^{3}H-progesterone from unbound hormone was carried out as described in the Materials and Methods of Chapter II. Radioactivity was counted in a liquid scintillation counter. The antibody dilution that enabled 50% of ^{3}H-progesterone to bind to the antibody was determined as adequate for future progesterone assays. The results are shown in Table G-1 and are expressed as cpm and as percent of total radioactivity added.

Table G-1. The effect of different dilutions of progesterone antibody on binding of ^{3}H-Progesterone; specific binding is the mean of three replicates

Antibody Dilution	Specific Binding (cpm)	Percent of Total Radioactivity
1:100	6260	70
1:250	5365	60
1:375	4516	50
1:500	4062	45
1:750	3635	40
1:1000	2972	33

APPENDIX H

Table H-1. Experiment I: serum progesterone levels in super-ovulated rats treated with hCG, GnRH and $PGF_{2\alpha}$ and determined at five hourly intervals for up to 25 hours

Treatment	Serum Progesterone (ng/ml)					
	0h	5h	10h	15h	20h	25h
Control	151 ±8	154 ±26	147 ±26	330 ±51	185 ±36	244 ±59
hCG	*	179 ±3	147 ±22	193 ±40	152 ±23	295 ±26
GnRH	*	110 ±11	72 ±6	207 ±100	101 ±34	216 ±100
$PGF_{2\alpha}$	*	73 ±29	46 ±21	131 ±21	112 ±48	87 ±48

Results are expressed as mean ± standard error of the mean (n=3).

*Treated animals not sacrificed at time 0.

Table H-2. Experiment II: serum progesterone levels in super-ovulated rats treated with hCG, GnRH and $PGF_{2\alpha}$ and determined every 24 hours for up to five days

Treatment	Serum Progesterone (ng/ml)					
	0d	1d	2d	3d	4d	5d
Control	128 ±55	75 ±10	58 ±22	66 ±8	73 *	53 ±1
hCG	**	86 ±14	42 ±10	50 ±12	52 ±33	37 ±19
GnRH	**	92 ±6	70 ±4	50 ±11	76 ±17	6 ±1
$PGF_{2\alpha}$	**	5 ±0.3	46 ±9	47 ±18	52 ±29	14 ±7

Results are expressed as mean ± standard error of the mean (n=3).

*One animal only.

**Treated animals not sacrificed at time 0.

Table H-3. Experiment III: serum progesterone levels in super-ovulated rats treated with hCG, GnRH and $PGF_{2\alpha}$ and determined every two days after treatment for up to eight days

Treatment	Serum Progesterone (ng/ml)					
	0h	6h	2d	4d	6d	8d
Control	123 ±17	198 ±5	124 ±24	189 ±131	98 ±75	47 ±35
hCG	*	246 ±43	70 ±72	128 ±73	90 ±57	34 ±38
GnRH	*	160 ±16	352 ±164	114 ±95	90 ±52	4 ±2
$PGF_{2\alpha}$	*	38 ±18	39 ±14	50 ±46	11 ±8	6 ±2

Results are expressed as mean ± standard error of the mean (n=5 for controls; n=4 for treatments).

*Treated animals not sacrificed at time 0.

VITA

Paul Gibson was born in Queanbeyan, New South Wales, Australia in 1953. He completed his formal secondary education at Forbes High School and graduated from Sydney University in 1977 with a Bachelor of Science degree with honors. He obtained a Diploma in Education from Sydney Teachers' College and taught at Cowra High School for four years prior to studying for a Master's degree in Zoology at The University of Tennessee, Knoxville, Tennessee.