The lipid composition of plasma membranes of corpora lutea was examined to determine whether changes in this organelle occur during regression and early pregnancy in the ewe. Forty ewes were assigned to be necropsied on days 13 or 15 of the estrous cycle (D13-NP and D15-NP) or pregnancy (D13-P and D15-P). Purification of luteal membranes on discontinuous sucrose gradients yielded two fractions that exhibited the greatest enrichment of 5'-nucleotidase activity over that of the homogenate, and the lowest contamination by endoplasmic reticulum and mitochondrial membranes. Predominant phospholipids identified in membranes obtained from all groups were phosphatidylcholine (PC, 48.9±0.6% of total phospholipid), phosphatidylethanolamine (PE, 33.3±0.4%), sphingomyelin (SPH, 9.7±0.3%), phosphatidylserine (PS, 3.5±0.2%) and phospha-
tidylinositol (PI 4.0±0.5%). No changes were observed in the µg phospholipid/mg membrane protein for any luteal phospholipid and in the free cholesterol to phospholipid ratio on days 13 and 15 of the estrous cycle or pregnancy. No significant changes in the relative percentages of the major fatty acids present in PC (16:0, 18:1), PE (18:0, 18:1, 20:4) or PS (18:0, 18:1, 22:4) nor in the ratios of unsaturated (U) to saturated (S) fatty acids in these phospholipids were observed. The profile of the major fatty acids present in PI revealed a decrease in 18:0 in D15-NP, an increase in 20:4 in D15-P and an increase in 22:4 in luteal membranes of both D13- and D15-NP ewes relative to levels of these fatty acids in PI of corresponding NP or P ewes. Specific binding of [125I]iodo-hCG to luteal plasma membranes from NP and P ewes on days 13 and 15 (6/group) revealed similar affinities and concentrations of unoccupied LH receptors. These results indicate that major changes in the gross composition of luteal plasma membrane lipids and LH receptor binding are not associated with corpus luteum maintenance during the time of maternal recognition of pregnancy in the ewe. However, changes in phospholipid unsaturated fatty acids of 20 or more carbon atoms may be indicative of continued luteal function during early pregnancy in the ewe.
Plasma Membrane Composition
and Luteinizing Hormone Receptors
of Ovine Corpora Lutea during Early Pregnancy
by
Mary Beth Zelinski

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PLASMA MEMBRANE COMPOSITION AND LUTEINIZING HORMONE RECEPTORS OF OVINE CORPORA LUTEA DURING EARLY PREGNANCY

REVIEW OF LITERATURE

Introduction

As early as 1573, the corpus luteum (CL) was identified as a distinct feature of the female ovary by Volcherus Coiter, a student of the anatomist Fallopius, yet its physiological role in the reproductive process was not realized until centuries later. The first documented experiments regarding luteal function were reported by Fraenkel (1903) and his researchers who observed that cauterization of CL in pregnant rabbits resulted in resorption of the embryos or abortion, thus establishing an essential nature of the CL for preparing the uterus for implantation and the maintenance of pregnancy. Corner and Allen (1929) were the first to isolate the steroid hormone, progesterone, from porcine CL and to identify it as the primary secretory product of the CL. These early researchers developed a bioassay for progesterone based upon their observations that treatment of ovariectomized rabbits with CL extracts resulted in proliferation of the uterine endometrium similar to that seen in pseudopregnant rabbits. Between 1930 and 1934, research by Allen, Hisaw,
Butenandt and Slottan led to the isolation of crystalline progesterone from CL and its eventual synthesis (reviewed by Corner, 1947). Subsequent studies established that the maintenance of pregnancy in the ovariectomized animal could be achieved by sufficient quantities of exogenous progesterone (Allen and Corner, 1930; Pincus et al., 1956; Foote et al., 1957). Thus, the main function of the CL appears to be the production of progesterone. Progesterone action in the uterus provides an appropriate milieu wherein conceptus development can occur. The continuous release of luteal progesterone is absolutely necessary for maintenance of pregnancy in the ewes, at least until day 55 of gestation (Casida and Warwick, 1945).

Maternal Recognition of Pregnancy in the Ewe

The average length of the estrous cycle in sheep is about 16±1 days; ovulation usually occurs within 24 hours after the onset of estrus (day 0) resulting in the formation of the CL. A discernible increase of progesterone in systemic plasma occurs on day 4, reaches a plateau by day 7-8, then rapidly declines by day 15 (reviewed by Robertson, 1977). A first requirement of pregnancy in the ewe is the prolongation of the life span of the CL by preventing the recurrence of ovulation. It is believed
that chemical signals originating from the embryo and its membranes prohibit the return to estrus and that synthesis of these signals constitutes one of the earliest events occurring between the ewe and the embryo. Short (1969) has described this early event as the "maternal recognition of pregnancy." Failure of the embryo to produce the appropriate signals, or failure of the ewe to respond to the presence of the embryo, results in the termination of pregnancy.

Knowledge of the regulation of luteal function by pituitary and uterine factors is paramount to understanding the mechanisms by which the presence of the embryo ensures maintenance of the corpus luteum. Endocrine control mechanisms underlying the ephemeral nature of the CL during the estrous cycle, and abrogation of luteal demise by the presence of the embryo will be discussed below.

Regulation of Luteal Function

Pituitary Regulation of Luteal Function

In 1905, Heape was the first to suggest that a substance he termed "generative ferment" circulated in the blood and controlled ovarian activity (see Corner, 1947). The pioneering work of Smith and Engle (1927) revealed the presence of such factors in the anterior pituitary glands
of rats because hypophysectomy caused the gonads to atrophy, while injections of pituitary extracts restored gonadal function. Fevold et al. (1931) and Evans et al. (1939) isolated the gonadotropic factor of the anterior pituitary gland, and called it luteinizing hormone (LH), based upon its predominant biological activity in the female, and interstitial-cell-stimulating hormone (ICSH) because of its tropic effects on these cells in the male testes. Subsequent studies confirmed that LH and ICSH were identical. The subsequent extraction and purification of LH in large quantities made it available for structural and biological studies then, and now.

The earliest attempts to define the pituitary requirements for normal luteal function in sheep have been summarized by Denamur (1974). Hypophysectomy of ewes 40 hours after the onset of estrus allowed some, although incomplete, luteal development. Denamur also observed that hypophysectomy on day 3 of the cycle did not impair luteal development over the subsequent 9 days post-surgery, but progesterone secretion was reduced between days 10 and 15 of the cycle. In contrast, Kaltenbach et al. (1968a) observed that the formation of CL in ewes hypophysectomized on the day of ovulation was completely prevented, while hypophysectomy of ewes on day 5 of the estrous cycle resulted in complete luteal regression by day 12. Although the data regarding CL formation following hypophysectomy immediately following ovulation
are at odds, removal of the pituitary between days 3 and 5 was detrimental to luteal function, indicating the necessity of this gland for progesterone secretion.

Subsequent experiments by Kaltenbach et al. (1968b) revealed that constant infusions of crude preparations containing LH and follicle-stimulating hormone (FSH) into ewes hypophysectomized and hysterectomized on day 12 of the cycle maintained CL weight and progesterone content to day 20, whereas infusion of prolactin (PRL) did not prevent CL regression. Similarly, Karsch et al. (1971) observed that constant infusions of LH, but not PRL, also prolonged luteal life span and function in uterine-intact, cycling ewes. These data suggested that LH was the luteotropic hormone responsible for ovine luteal function during the estrous cycle.

Hypophysectomy on day 3 or 10 of pregnancy caused complete luteal regression and abortion by day 20 (Denamur, 1974). Similar effects are observed in ewes hypophysectomized on day 30; however, if this surgery is performed on day 60, pregnancy can be maintained (Denamur, 1974). Continuous infusions of the crude LH-FSH preparation, but not PRL, maintained progesterone secretion and a viable embryo in hypophysectomized pregnant ewes (Kaltenbach et al., 1968b). These data again support the idea that LH is absolutely required for the maintenance of pregnancy until midgestation.

Daily injections of LH antiserum to cycling ewes
caused luteal regression (Fuller and Hansel, 1970).
Injection of anti-ovine LH serum into nonpregnant ewes on
day 9 reduced CL weight and progesterone secretion within
12 hours post-injection (Niswender et al., 1981).
Furthermore, LH enhances the secretion of progesterone
from ovine CL in vivo (Niswender et al., 1976) and in vitro
(Kaltenbach et al., 1967; Simmons et al., 1976). Thus, it
is currently accepted that the most important factor
involved in regulating luteal function is LH.
The early observations by Denamur (1974) also
suggested a luteotropic role for PRL in the ewe, because
injections of PRL into hypophysectomized and hysterec-
tomized ewes maintained luteal function. However, it must
be noted that the "luteotropic" effects of PRL were only
manifested in hysterectomized ewes, whereas PRL was
without effect in uterine-intact ewes. In addition, these
experiments also failed to show a luteotropic effect of LH
in nonpregnant and pregnant ewes, but this could be due to
the fact that the LH was injected (Denamur, 1974), while
only constant infusions of LH were effective (Kaltenbach
et al., 1968b). The extent of contamination of the PRL
preparations with other anterior pituitary hormones was
also unknown, although the study by Karsch et al. (1971)
indicated that PRL was not luteotropic in uterine-intact,
hypophysectomized ewes. Subsequent studies revealed that
injections of 2-Br-a-ergocryptine, which reduced serum PRL
levels by 95% over the entire estrous cycle, alone or in
combination with PRL antiserum had no effect on estrous cycle length or serum progesterone levels (Niswender, 1974; Niswender et al., 1976). Infusions of PRL into the ovarian artery of ewes did not increase progesterone secretion (McCracken et al., 1971). Dispersed ovine luteal cells did not respond to PRL treatment in vitro (Simmons et al., 1976). Thus, the available evidence, although still controversial, does not support a luteotropic role of PRL in the ovine CL.

Uterine Regulation of Luteal Function

Demonstration that hysterectomy prolonged CL function in the ewe led to the implication that the uterus plays a major role in the regulation of luteal life span in this species (Wiltbank and Casida, 1956). A local luteolytic effect of the uterus appeared to exist since unilateral hysterectomy of ewes resulted in CL regression in ovaries ipsilateral to the intact horn while those on the contralateral side were maintained (Inskeep and Butcher, 1966). Various experiments employing autotransplantation of reproductive tracts to the necks of ewes further verified the presence of a local effect of the uterus in regulating CL life span (McCracken et al., 1971). Subsequent to the suggestion that prostaglandin $F_2\alpha$ (PGF$_2\alpha$) may be the uterine luteolysin based upon its relative abundance in the uterus (Pharris and Wyngarden, 1969), McCracken et al. (1972) proved that PGF$_2\alpha$ was indeed the luteolysin in the
ewe. Uterine venous blood from a donor ewe on day 15 of
the cycle infused into the arterial supply of the trans-
planted ovary of a recipient ewe induced luteal regression
similar to that observed with intraarterial infusion of
PGF$_2$$\alpha$ (McCracken et al., 1972).

Separation of the ovarian artery from the utero-
avarian vein prevented regression of the ovine CL,
suggesting that the luteolysin in uterine venous blood
reached the ovary via a countercurrent mechanism between
this vein and the adherent ovarian artery (Barrett et al.,
1971). McCracken et al. (1972) confirmed this route of
transport by demonstrating that infusion of tritium-
labeled PGF$_2$$\alpha$ into the uterine vein resulted in a greater
amount of radioactivity present in the ovarian arterial
than in iliac arterial blood. Anastomoses of uterine
veins or arteries in unilaterally hysterectomized ewes
showed that uterine-induced luteal regression is exerted
through a local veno-arterial pathway between a uterine
horn and its adjacent ovary (Ginther et al., 1973).
Anatomical studies beautifully demonstrated areas of close
apposition between the uterine vein and the tortuous
tightly-coiled ovarian artery (Ginther and Del Campo,
1973) where the direct passage of PGF$_2$$\alpha$ most likely
occurred. A subsequent study utilizing various surgical
anastomoses confirmed the adequacy of the main uterine
vein as an "outlet" for the luteolysin and the ovarian
artery as the final component of the pathway from uterus
to ovary (Mapletoft and Ginther, 1975). The precise nature of the transfer of PGF₂ₐ from one vessel to the other is not fully understood, but perhaps involves diffusion through the intercellular spaces of the intervening vessel walls (Ginther, 1974).

Copious literature exists concerning uterine secretion of PGF₂ₐ during the ovine estrous cycle, and has been thoroughly reviewed by Inskeep and Murdoch (1980). Sampling of endometrial tissue, jugular or utero-ovarian venous concentrations of the luteolysin during the estrous cycle has revealed increased levels on day 13 to 14 with maximal pulsatile release (five pulses, each lasting one hour, over a 24-hour period) occurring on days 14 to 15 and lasting until the onset of estrus (Inskeep and Murdoch, 1980; McCracken et al., 1984). The principal site of PGF₂ₐ secretion is the uterine endometrium (Wilson et al., 1972), where both caruncular and intercaruncular cell types contribute to its synthesis (Huslig et al., 1979).

The endocrine regulation of PGF₂ₐ secretion during luteolysis has been summarized by McCracken et al. (1984), and appears to be controlled primarily by estradiol and oxytocin. Administration of exogenous estradiol on days 11 or 12 of the estrous cycle induces premature luteal regression in intact, but not hysterectomized ewes (Stormshak et al., 1969; Hawk and Bolt, 1970). Exogenous estradiol also stimulates de novo PGF₂ₐ secretion from the
ovine endometrium only during the later stages of the estrous cycle (Barcikowski et al., 1974; Ford et al., 1975) and this effect can be blocked by indomethacin, an inhibitor of prostaglandin synthesis. Destruction of follicles by electrocautery or X-irradiation resulted in heavier CL on day 17 (Ginther, 1971) and a delay in CL regression (Karsch et al., 1970). A close temporal relationship between estradiol and PGF$_2$a during the final stages of luteolysis, but not on days 12 to 13, has been observed (Inskeep and Murdoch, 1980).

Roberts et al. (1975) showed that intra-arterial infusions of oxytocin were also capable of evoking PGF$_2$a secretion during the late luteal stage. Oxytocin-induced PGF$_2$a secretion was presumed to occur through an oxytocin-receptor interaction since the highest concentration of endometrial oxytocin receptors were observed during the late luteal phase (Roberts et al., 1976). Immunization against oxytocin prolongs the luteal phase in ewes (Sheldrick et al., 1980). Recently, it has been shown that the ovine CL contains high concentrations of oxytocin (Watkins, 1983) which is secreted together with its associated neurophysin into the ovarian vein (Watkins et al., 1984). Pulsatile surges of oxytocin and oxytocin-associated neurophysin occur during luteolysis in the ewe (Fairclough et al., 1980; Flint and Sheldrick, 1983) and have confirmed the CL, rather than the posterior pituitary gland, as the major source of the surges (Moore et al.,
1986). The observations that exogenous oxytocin does not cause luteal regression in hysterectomized ewes (Hatjimaiooglou et al., 1979), and that increases in utero-ovarian levels of PGF$_2$-a are observed prior to oxytocin pulses on day 15 (Moore et al., 1986), suggest that uterine PGF$_2$-a initiates oxytocin release from the CL during the later stages of luteolysis.

Two similar hypotheses to explain the sequence of events regulating luteolysis in ovine CL have been suggested (McCracken et al., 1984; Flint and Sheldrick, 1985) and will be summarized. It should be noted that there is no consensus as to the precise mechanism causing the initial, early release of PGF$_2$-a from the uterus. However, McCracken et al. (1984) postulate that because the uterotrophic actions of progesterone appear to decline as the luteal phase progresses, endogenous estradiol is now able to stimulate oxytocin receptor synthesis in the endometrium. Endogenous oxytocin, presumably of luteal origin, interacts with its receptor to cause secretion of PGF$_2$-a from the endometrium. Luteal regression is initiated as a result of the countercurrent transfer of PGF$_2$-a from the uterine vein to the ovary artery. Further release of oxytocin from the CL is caused by PGF$_2$-a, and oxytocin binding to the endometrium further reinforces PGF$_2$-a release in a positive feedback manner. This later release of luteal oxytocin appears to cause the pulsatile secretion of PGF$_2$-a on days 14 to 15. Since oxytocin
receptors may be desensitized for a period of time subsequent to oxytocin binding, hour-long pulses of endometrial PGF$_2\alpha$ release occur every six hours, which is the time necessary for estradiol to induce the synthesis of new oxytocin receptors. Clearly, this hypothesis is open to further scrutiny, but summarizes, based on available data, the mechanisms whereby endogenous estradiol and oxytocin regulate PGF$_2\alpha$ release during luteolysis in the ewe.

The mechanisms by which PGF$_2\alpha$ induce CL regression are not well defined, "despite a plethora of studies by impressive minds in the field" (Inskeep and Murdoch, 1980). Two theories have emerged to support vascular effects of PGF$_2\alpha$ on ovarian or luteal blood flow, first suggested by Warbritton (1934), and biochemical events occurring directly in the luteal cell (Henderson and McNatty, 1975). The latter proposal regarding the biochemical processes whereby PGF$_2\alpha$ inhibits LH-induced progesterone production in luteal cells during luteolysis will be discussed in the final chapter after a presentation of the cellular mechanism of action of these hormones.

Niswender et al. (1976) present a comprehensive review on the role of blood flow as a mediator of ovarian function. It was shown that ovarian arterial blood flow to the ovary bearing a CL was positively correlated with serum progesterone levels during the estrous cycle, and decreased blood flow to the luteal ovary occurred during
the time of luteolysis. These changes in ovarian blood flow were also reflected by a linear increase in the uptake of radioactive microspheres by CL from days 2 through 10 of the cycle, and a dramatic decline from days 12 through 16. The CL was shown to receive the majority of the blood. In the absence of a CL, ovarian blood flow and that observed in nonluteal ovarian compartments did not change throughout the cycle. It was also demonstrated that LH, but not PRL, could increase ovarian blood flow, although this increase was not as dramatic as the elevation in systemic progesterone following LH infusion. Treatment of ewes with PGF₂α on day 5 caused a decrease in ovarian luteal blood flow within 4 hours, and a decline in systemic progesterone levels by 6 hours, while both remained depressed for 24 hours. As a result, in the decline of luteal blood flow, necessary blood-borne substances required for maximal steroidogenesis (oxygen, glucose, cholesterol, LH) would be limited from gaining access to the CL. Debris in the lumen of luteal capillaries, apparently a result of endothelial cell degeneration, appeared coincidentally with morphological evidence of decreased luteal cell viability during natural (O'Shea et al., 1977) and PGF₂α-induced regression (Chamley and O'Shea, 1976). Thus, the dramatic hemodynamic changes reflected mainly by decreased luteal blood flow occurring within hours after PGF₂α administration indicate that this
may be a part of the mechanism involved in luteal regression.

Two Cell Model for Regulation of Luteal Function

More than fifty years ago, Warbritton (1934) described the presence of two distinct cell types in the ovine CL, which are presently referred to as large and small cells based upon their most distinguishing characteristic of cell diameter. Additional investigators confirmed the existence of large and small cells in ovine (Deane et al., 1966; O’Shea et al., 1979; Rodgers and O’Shea, 1982; Niswender et al., 1985b), bovine (Foley and Greenstein, 1958; Uresley and Leymarie, 1979; Koos and Hansel, 1981) and porcine (Corner, 1919; Lemon and Loir, 1977) CL and compared their morphological, biochemical and functional characteristics. Table 1 provides a brief summary of the comparative properties describing the large and small luteal cells of the ewe.

The small luteal cells are classified on the basis of their diameter ranging from 12–22 μm, spindle shape, large lipid droplets, and irregularly shaped nuclei that appear to contain cytoplasmic inclusions distinct from the nucleolus. The large luteal cells range from 23–35 μm in diameter, have a more regular round cellular and nuclear shape, and appear to be less numerous than small cells, comprising approximately 30% of the CL on a volume basis and 25–30% of the luteal cells staining positively for
Table 1. Morphological and biochemical properties of the large and small cells of ovine corpora lutea.

<table>
<thead>
<tr>
<th>Property</th>
<th>Large Cells</th>
<th>Small Cells</th>
<th>References(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>22-35 µm</td>
<td>12-22 µm</td>
<td>O'Shea et al., 1979</td>
</tr>
<tr>
<td>% CL volume</td>
<td>30</td>
<td>16</td>
<td>Niswender et al., 1976</td>
</tr>
<tr>
<td>% HSD&lt;sup&gt;a&lt;/sup&gt; positive cells</td>
<td>25-30</td>
<td>70-75</td>
<td>Niswender et al., 1985</td>
</tr>
<tr>
<td>Assumed follicular origin</td>
<td>granulosa cells</td>
<td>thecal walls</td>
<td>O'Shea et al., 1980</td>
</tr>
<tr>
<td>Cell shape</td>
<td>regular</td>
<td>spindle, elongated</td>
<td>O'Shea et al., 1979</td>
</tr>
<tr>
<td>Nuclear shape</td>
<td>round, no inclusions</td>
<td>irregular shape,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>contain cytoplasmic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>inclusions</td>
<td></td>
</tr>
<tr>
<td>Plasma membrane surface</td>
<td>microvilli</td>
<td>smooth</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enders and Lyons, 1973</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>present</td>
<td>present</td>
<td>Deane et al., 1966;</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>numerous</td>
<td>numerous</td>
<td>O'Shea et al., 1979</td>
</tr>
<tr>
<td>Lipid droplets</td>
<td>absent</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>present</td>
<td>absent</td>
<td></td>
</tr>
<tr>
<td>Secretory granules</td>
<td>present</td>
<td>absent</td>
<td>Gemmell et al, 1974, 1976</td>
</tr>
<tr>
<td>Basal Pb</td>
<td>42.3±29.7</td>
<td>2.2±1.0</td>
<td>Fitz et al., 1982</td>
</tr>
<tr>
<td>LH-stimulated P</td>
<td>50.8±35.7</td>
<td>27.3±8.8</td>
<td></td>
</tr>
<tr>
<td>dibutyryl cAMP-stimulated P</td>
<td>28.3±21.7</td>
<td>19.7±4.3</td>
<td></td>
</tr>
<tr>
<td>oLH RC</td>
<td>3100±5300</td>
<td>33000±13900</td>
<td></td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; R</td>
<td>11000±2400</td>
<td>900±1100</td>
<td></td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; R</td>
<td>68000±14700</td>
<td>2100±1000</td>
<td></td>
</tr>
<tr>
<td>Estradiol R</td>
<td>8100±2000</td>
<td>1000±300</td>
<td>Glass et al., 1985</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Property</th>
<th>Large Cells</th>
<th>Small Cells</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to Forskolin and cholera toxin</td>
<td>none</td>
<td>increase P</td>
<td>Hoyer et al., 1984</td>
</tr>
<tr>
<td>cAMP-bound to protein kinase</td>
<td>present</td>
<td>present</td>
<td>Hoyer and Niswender, 1985</td>
</tr>
<tr>
<td>Response to protein kinase activation</td>
<td>none</td>
<td>increase P</td>
<td>&quot;</td>
</tr>
<tr>
<td>Response to exogenous 25 hydroxycholesterol</td>
<td>none</td>
<td>increase P</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

\(^{a}\text{HSD} = 3\beta\text{-hydroxysteroid dehydrogenase}\)

\(^{b}\text{P} = \text{progesterone production, Fg/cell/min; mean} \pm \text{SEM}\)

\(^{c}\text{R} = \text{receptor sites/cell; mean} \pm \text{SEM}\)
3β-hydroxysteroid dehydrogenase activity (the enzyme that converts pregnenolone to progesterone). Large cells also display a prominent basal lamina containing numerous microvillous folds on the cell surface. Both cell types possess numerous mitochondria with tubular cristae and abundant smooth endoplasmic reticulum, the hallmarks of active steroid-secreting cells. The large luteal cells characteristically exhibit the presence of an extensive rough endoplasmic reticular network and numerous membrane-bound secretory granules consistent with a protein secretory function, while these organelles are conspicuously absent from the small cell cytoplasm.

The large luteal cell secretory granules are distinct from lysosomes because they lack acid phosphatase and catalase activity (Gemmell et al., 1974; McClellan et al., 1977) and are released at the plasma membrane by exocytosis (Gemmell et al., 1974; Sawyer et al., 1979). Secretory granule release was shown to occur concomitantly with progesterone secretion, which led several investigators to suggest that the granules contained progesterone (Gemmell et al., 1974; Sawyer et al., 1979) and/or a progesterone-binding protein (Gemmell and Stacy, 1979; Quirk et al., 1979). Because numerous attempts to isolate purified populations of luteal secretory granules devoid of smooth endoplasmic reticulum failed, McClellan et al. (1979) pulse-labeled ovine luteal slices with tritiated pregnenolone to determine the intracellular localization
of progesterone by electron microscopy and autoradiography. At all time periods studied, secretory granules were devoid of labeled progesterone. Similarly, Sernia et al. (1982) failed to observe both the presence and specific binding of progesterone in a morphologically intact, enriched granule fraction obtained by sucrose gradient centrifugation. Thus, the current evidence does not support the hypothesis that progesterone is sequestered and transported in these secretory granules. Based upon data indicating that progesterone can penetrate into synthetic liposomes consisting of dipalmitoylphosphatidylcholine and cholesterol, Carlson et al. (1983) suggested that progesterone may leave the luteal cell by simple diffusion. Recently, indirect evidence in support of the proposition that the secretory granules may contain oxytocin has shown the immunocytochemical localization of oxytocin and its associated neurophysin to be limited to the large luteal cells in ewes (Watkins, 1983; Sawyer et al., 1986). Furthermore, luteal oxytocin was found to be stored almost exclusively in the large cells of ovine CL, and the large cells were capable of oxytocin synthesis in vitro whereas the small cells lacked the ability to produce measurable quantities of this peptide (Rodgers et al., 1983b). Data directly displaying the presence of oxytocin in secretory granules from large ovine luteal cells is lacking; however, Fields et al. (1986) have shown neurophysin immunoreactivity localized to these
organelles in bovine large luteal cells. Luteal secretory granules have been directly shown to contain relaxin in bovine (Fields et al., 1980) and porcine CL (Fields and Fields, 1985). Thus, while the existence of secretory granules capable of exocytosis in large luteal cells is undisputed, the nature of their contents appears to be represented by numerous peptides.

It is now generally accepted that both the granulosa and theca cells of the ovulatory follicle become incorporated into the CL of many mammalian species, yet the precise follicular origin of the large and small luteal cells remains controversial. It has been technically difficult to follow the fate of these cell types subsequent to ovulation because the once-prominent basal lamina separating granulosa from theca interna cells vanishes within 24 hours, bringing both cell populations into close apposition with subsequent dispersal within the CL by 48 hours (O'Shea et al., 1980). In addition, there are no known genetic, antigenic and/or biochemical markers exclusive to either cell type that could be monitored during the genesis of the CL. In the ovine follicle, alkaline phosphatase activity is limited to the theca interna, and O'Shea et al. (1980) monitored the distribution of this enzyme at various times post-ovulation in ewes using a histochemical staining procedure. These researchers provided the only direct evidence to date that the small luteal cells in sheep are derived from the theca interna
as indicated by their positive staining, whereas the large cells arise from granulosa cells based upon ultrastructural comparisons within the first 24 hours post-ovulation. The distinct origins of the large and small cells was only evident within the first 48 hours after ovulation because at later times the reaction for alkaline phosphatase became decreased and less consistent, which may have been due to thecal cell migration and/or acquisition of this enzyme by the granulosa-derived cells as the CL matured.

An innovative method involving the use of two monoclonal antibodies, one that recognized a theca-specific antigen and the other a granulosa-specific antigen, in an indirect immunofluorescence assay was recently developed by Alila and Hansel (1984) to monitor the fate of these cell populations during the luteinization of bovine CL. Early in the estrous cycle, from days 4 to 6, 77% of the large luteal cells bound the granulosa antibody, while 70% of the small cells bound the theca antibody, thus tending to confirm the data from the ewe that small and large cells arise from theca and granulosa cells, respectively. However, as the cycle progressed, the proportion of large cells displaying fluorescence upon binding to the granulosa antibody decreased, while a greater proportion of large cells now bound the theca antibody, and the small cells still exhibited a reaction only with the theca antibody. However, there are several possible explana-
tions for why more of the large cells displayed a positive reaction with the theca antibody: 1) as the large, granulosa-derived cells mature, they lose the specific antigen that may have been present while in the follicle prior to ovulation; 2) the large, granulosa-derived cells have a limited lifespan of approximately 4 days; and/or 3) during luteal maturation, the small cells may differentiate into large cells, as first proposed by Donaldson and Hansel (1965). The source of the antigens used to produce the monoclonal antibodies were bovine follicular theca interna plasma membranes and rat granulosa cells, and they were not further characterized beyond their cellular origin. Thus, it is difficult to ascertain whether they represent distinct populations of antigens or are directed against molecules common to both cell types that may not be expressed during the entire life span of the bovine CL.

Evidence to substantiate the possible development of small cells to large cells in ovine CL is meager at best, and is based upon circumstantial morphometric analysis of CL throughout the estrous cycle. In one study analyzing dissociated luteal cells, Niswender et al. (1985b) reported the total number of steroidogenic (HSD-positive), large and small cells increased approximately four-fold between days 4 and 8 of the estrous cycle, then declined through day 16. When characterizing a center slice of ovine CL by electron microscopy, data from the same laboratory revealed a similar distribution of steroido-
genic and small cells during the estrous cycle, but in contrast, the number of large luteal cells remained constant from days 4 through 16 (Farin et al., 1986). These researchers did note that although the size of the small cell, as measured by area, was unaltered throughout the cycle, the cellular area of the large cells increased from day 4 to day 16.

Although the data concerning the large cells are apparently conflicting, these results can be explained by the speculative model described by Niswender et al. (1985b). One basic premise of the model is the existence of a population of nonsteroidogenic stem cells within the ovine CL, capable of giving rise to the small cells which in turn develop into large cells possessing a limited life span. If the stem cells became depleted and/or their transformation to small cells became inhibited by some hormonal mechanism, luteal regression would result.

One piece of indirect evidence to support the presence of stem cells indicates that although the granulosa-derived (large) luteal cells do not exhibit mitosis after ovulation, stromal cells represented by endothelial and fibroblastic cells did undergo secondary mitosis on day 2-3 post-ovulation in the ewe (McClellan et al., 1975). The relative proportion per CL of a nonsteroidogenic (HSD-negative) population of ovine luteal cells, characterized by a diameter of 8 μm, increased between days 4 and 8 of the estrous cycle (Niswender et al., 1985b).
which directly parallels the change in the number of small luteal cells. Cells intermediate in size between fibroblasts and small cells were also observed by O'Shea et al. (1980). The nonsteroidogenic, putative stem cell population acquires the capacity to synthesize progesterone most likely under the luteotropic influence of LH and would then give rise to small cells up until day 8. The differentiation of small cells into large cells is supported, albeit indirectly, by observations of a range of cell sizes, rather than two absolutely discrete diameters, within the ovine CL and by injection of hCG into ewes on day 8 of the estrous cycle which resulted in an increased ratio of large to small cells in CL collected on day 10 (Niswender et al., 1985b). Presumably, the population of small cells, present through at least day 12 of the cycle while under the influence of tonic circulating LH concentration, is the source of the large cells. Because the proportion of large cells remains constant, there most likely is not a one-to-one conversion of small-to-large cells and/or this may reflect a dynamic balance between the development and degeneration of large cells. The decline in the number of small cells between days 8 and 16 could possibly be reflected by the cessation of stem cell mitoses, in combination with the reduction in blood flow to the CL during luteolysis. Obviously, much more research is necessary to substantiate the mechanisms involved in ovine luteal cell population dynamics.
throughout the estrous cycle.

It is interesting to note that in bovine CL, only the small cells persisted throughout pregnancy while the large cells disappeared by day 100 post-mating (Alila and Hansel, 1984). Similar morphological observations indicated a progressive deterioration of large cells with advancing pregnancy in the cow, with no apparent change in small cell structure (Fields et al., 1985). Possible changes in luteal cell populations during pregnancy in the ewe have not yet been determined. Further examination of luteal morphology, correlated with function, may provide interesting information concerning the mid-pregnancy transition from pituitary/embryonic regulation of progesterone synthesis to placental steroidogenesis in the ewe, as well as comparative insights into the necessity for pituitary regulation for the duration of pregnancy in the cow.

In addition to the differences in morphology, large and small luteal cells are also distinct with regard to several of their biochemical properties. Although both cell types secrete progesterone, recent studies have indicated that the regulation of steroidogenesis in large and small cells appears to be dissimilar in a number of respects.

Luteinizing hormone causes a marked stimulation, approximately 20-fold, of progesterone secretion from small ovine luteal cells (Fitz et al., 1982; Rodgers
et al., 1983). Conversely, progesterone secretion from large luteal cells is largely unaffected by LH. The stimulatory action of LH in small cells is congruent with the observation that the numbers of unoccupied LH receptors/cell were 10-fold greater than those in large cells (Fitz et al., 1982). The general assumption that LH is the primary regulator of progesterone secretion in the ovine CL would imply that the small luteal cells would synthesize the majority of luteal progesterone. However, in the absence of LH, large cells secrete at least four times more progesterone than small cells (Koos and Hansel, 1981; Fitz et al., 1982; Rodgers et al., 1983a). Because the number of small cells decreases between days 8 and 16 of the cycle while the number of large cells remains constant, it is likely that large cells should also make a significant contribution to luteal progesterone secretion. Niswender et al. (1985b) calculated that the contribution of small cells to the total amount of progesterone secreted over a 4-hour period during the mid-luteal phase in ewes ranged from 12 to 63%, with a total contribution of 22%. Based upon these calculations, they conjectured that most of the progesterone secreted by the ovine CL is from large cells which are unresponsive to LH in vitro.

Exposure of small and large ovine luteal cells to 25-hydroxycholesterol, a cholesterol analogue that freely diffuses into steroidogenic cells and is utilized as a
substrate for progesterone synthesis at a point beyond the rate limiting step in steroidogenesis, was used to assess the maximal rate of steroid production by the two cell types (Hoyer and Niswender, 1985). Although maximal rate progesterone production in small and large cells was similar in the presence of 25-hydroxycholesterol, a 36-fold increase in progesterone secretion over basal levels was observed in small cells whereas only a 7-fold increase over basal was exhibited by large cells. These results could be interpreted to mean that progesterone secretion in large cells under basal conditions is nearer to a maximal rate than in small cells.

Exposure of small ovine luteal cells to dibutyryl cAMP, as well as to agents which activate adenylate cyclase (forskolin and cholera toxin) stimulated progesterone secretion (Fitz et al., 1982; Hoyer et al., 1984). These data suggest that progesterone production by small cells is regulated by the cAMP second messenger system (which will be discussed further in a following chapter). In contrast, dibutyryl cAMP, forskolin and cholera toxin were without effect on progesterone secretion from large cells although forskolin and cholera toxin did increase intracellular levels of cAMP in large cells (Hoyer et al., 1984). Both large and small cells contain a cAMP-dependent protein kinase (Hoyer and Niswender, 1985). When intracellular levels of cAMP are increased and a concomitant increase in cAMP bound to protein kinase in
both cell types is observed, progesterone secretion from small cells is increased whereas that from large cells is unaffected (Hoyer and Niswender, 1985). These results provide further support to the hypothesis that progesterone secretion from small cells is cAMP-dependent while steroidogenesis by large cells is regulated by a cAMP-independent mechanism.

Hoyer and Niswender (1985) also observed that exposure of small cells to 25-hydroxycholesterol resulted in increased progesterone production, whereas ram serum and cholesterol were ineffective. None of these additions stimulated progesterone secretion in large cells, providing evidence that the lack of responsiveness to cAMP was not due to a limitation of substrate available for steroidogenesis. It must be noted, however, that the relative degree of luteal uptake of these agents by each cell type was not assessed (Hoyer and Niswender, 1985).

Thus, it is clearly evident that progesterone synthesis in large cells is regulated in a different manner than in small cells. The nature of the mechanism(s) underlying the high basal rate of steroidogenesis in large cells was postulated by Hoyer and Niswender (1985) to include the following possibilities. Irreversible post-translational modification of phosphoprotein substrates directly involved in progesterone synthesis could occur, possibly during the proposed differentiation of small cells to large cells, thus permanently "turning
on" the steroidogenic pathways in the large cell. Alternatively, transcriptional and/or translational modifications in large cells as compared to small cells could result in increased levels of steroidogenic enzymes or regulator proteins. Investigation of these proposed mechanisms should prove interesting.

Large ovine luteal cells also display increased numbers of receptors for PGF$_2$α and PGE$_2$ as compared to small cells (Fitz et al., 1982). Progesterone secretion by large, but not small, luteal cells was stimulated by PGE$_2$ and did not appear to involve activation of adenylate cyclase or increased intracellular cAMP concentrations (Fitz et al., 1984a). Exposure of large luteal cells to PGF$_2$α in vitro resulted in a dose-dependent reduction of progesterone accumulation in the media, whereas PGE$_2$ elicited a biphasic dose-dependent increase in progesterone secretion (Fitz et al., 1984b). Small luteal cells appeared to show no response to PGF$_2$α (Fitz et al., 1984a). Additional morphological evidence revealed that the continuous exposure of large cells attached to culture plates to PGF$_2$α resulted in the disappearance of plasma membrane processes, the formation of smooth surfaces over extensive areas of the cells, and the acquisition of a "halo" of apparently extruded cytoplasmic contents (Fitz et al., 1984b). Unfortunately, morphological results of plated large cells continuously exposed to PGE$_2$ were unpublished, but it was stated that cell viability and
shape was similar to cells incubated without prostaglandins. These authors postulated that the large cells may release a "toxin" in response to PGF₂α which could diffuse to affect small cells, but must accumulate to "critical" levels before affecting large cell morphology and function.

Compiling the known data regarding the functional aspects of the large and small cells of ovine CL, Silvia et al. (1984a) proposed a hypothetical two-cell model to depict the regulation of the CL during luteolysis and early pregnancy. In the nonpregnant ewe, the tropic effects of LH during the luteal phase are manifested in the small cells through the stimulation progesterone secretion by a cAMP-dependent mechanism in addition to causing their differentiation into large cells that secrete the majority of luteal progesterone by an as yet unknown cellular mechanism. As endometrial production of PGF₂α increases during the late luteal phase and reaches the CL by countercurrent transfer from the uterine vein to the ovarian artery, the large luteal cells bind the luteolysin by virtue of their large population of PGF₂α receptors. The effects of PGF₂α on large luteal cells may involve the release of a substance(s) that acts on small luteal cells to disrupt the ability of LH to stimulate progesterone production. The nature of the large cell product is unknown, but Niswender et al. (1985b) mention unpublished results indicating that oxytocin of large cell
origin inhibits LH-stimulated progesterone secretion by small cells. Flint and Sheldrick (1985) summarize evidence against an intraluteal action of oxytocin based upon the controversial existence of luteal oxytocin receptors and lack of inhibition of progesterone synthesis by ovine luteal cells in vitro. However, an alternative indirect mechanism whereby the luteolytic action of PGF$_{2\alpha}$ on the large cells is extended to the small cells could involve luteal oxytocin release from the large cells that would act on the uterine endometrium to potentiate PGF$_{2\alpha}$ release sufficient to cause a reduction in ovarian blood flow, ultimately leading to decreased small cell function (Flint and Sheldrick, 1985). Upon longer exposure to the action of PGF$_{2\alpha}$, the proposed cytotoxic effects would be manifested upon the large cells themselves, thus leading to the functional and structural demise of the CL.

During early pregnancy, uterine secretion of PGF$_{2\alpha}$ remains elevated; however, PGE$_2$ of uterine and/or conceptus origin is secreted in greater amounts than in nonpregnant ewes. Luteolysis may be prevented by the action of PGE$_2$ on large luteal cells whereby the release of cytotoxic substances would be prevented, thus allowing LH-stimulated progesterone in small luteal cells to continue, viability of large cells to be maintained, and stimulation of progesterone synthesis in large cells to occur. Again, vasodilatory effects of PGE$_2$ may counteract the vasoconstrictive effects of PGF$_{2\alpha}$ to maintain adequate
blood flow to the ovary.

Although many parts are speculative, the two-cell model of the regulation of luteal function in domestic animals has provided, and will continue to provide, an interesting framework on which to base experimentation concerning the cellular mechanisms of action of lutetropic and luteolytic agents. A more detailed description of the interactions between LH and prostaglandins at the cellular level will be provided in the last chapter of this review.

Effects of the Conceptus on Uterine and Luteal Function

Maintenance of early pregnancy in the ewe requires that the CL remain functional, but the mechanism(s) whereby the presence of the conceptus prevents luteolysis may be complex. The classical research of Moor and Rowson (1966a) demonstrated that luteal function was markedly extended in ewes whose conceptuses are removed on day 13, 14 or 15, yet removal of the conceptus through day 12 resulted in normal luteolysis. Embryo transfer experiments indicated that a conceptus must be present in the uterus by day 12 for luteal function and pregnancy to be maintained (Moor and Rowson, 1966b). Transfer of conceptuses to nonpregnant recipient ewes on days 13 or 14 had a slight, or no effect on preventing luteolysis. Collectively, these data indicate that the critical time for maternal recognition of pregnancy in the ewe is between
days 12 and 13, which is 5 days prior to the attachment of the trophoblast to the endometrium (Amoroso, 1952). The presence of the conceptus for only 24 hours during the critical time can result in luteal maintenance for up to 25 days post-estrus (Moor et al., 1966a,b). Although luteolysis can be prevented in nonpregnant ewes if hysterectomy is performed as late as day 15, even after some functional and morphological regression has occurred (Moor et al., 1970), it appears that the ovine conceptus can only prevent the initial changes associated with luteolysis, but cannot halt the lytic changes once they have begun (Moor et al., 1966a,b).

The relationship between the day 12 to 13 conceptus and the CL appears to involve regulation of a local manner. Conceptuses transferred to a surgically isolated uterine horn ipsilateral to the CL resulted in luteal maintenance, whereas luteolysis occurred when conceptuses were transferred to the horn contralateral to the CL (Moor and Rowson, 1966c). Similarly, in ewes with CL in both ovaries, surgical separation of both horns and transfer of a conceptus to one horn resulted in CL maintenance only on the gravid side (Moor and Rowson, 1966c). Therefore, luteal maintenance in ovine pregnancy occurs via a unilat- eral effect of the conceptus under these experimental conditions. A single conceptus transferred to sheep with intact uteri and unilateral or bilateral CL can maintain luteal function in one or both CL irrespective of the
uterine horn in which they were placed (Niswender and Dziuk, 1966). This occurs because fluids and/or embryonic tissues can pass directly from the gravid horn to the nongravid horn through the common uterine body.

The precise nature of the conceptus signal(s) which maintains luteal function and its mechanism of action remains controversial. One line of evidence supports the hypothesis that the active substance derived from the conceptus is a protein with antiluteolytic properties. Attempts to characterize this signal revealed that homogenates of day 14 to 15 conceptuses maintained luteal function for one month or longer when infused daily into the uterine lumen of nonpregnant ewes (Rowson and Moor, 1967; Martal, 1979; Ellinwood, 1979a). Homogenates prepared from older conceptuses were ineffective in prolonging CL life span. Exposure of day 12 to 15 conceptus homogenates to heat or proteolytic enzymes destroyed their ability to maintain CL. It was also apparent that the proteinaceous signal(s) must be introduced into the uterine lumen in order to exert their effects since ovine conceptus homogenates did not demonstrate any capability to stimulate progesterone production by CL in vitro (Ellinwood et al., 1979a). This point is still controversial because Godkin et al. (1978) reported that ovine conceptus homogenates could enhance in vitro steroidogenesis by luteal cells. However, Ellinwood et al. (1979a) failed to demonstrate any PRL, LH or hCG
activity in these homogenates using radioreceptor assays.

Ovine blastocysts between 13 and 21 days of age incubated in vitro in the presence of tritiated leucine were shown to release proteins into the media at a linear rate over a given 24-hour period (Godkin et al., 1982). Day 16 blastocysts converted 8% of the radioactivity into non-dialysable macromolecules released into the media. At day 13, only one major protein was revealed by two-dimensional polyacrylamide electrophoresis. This protein, subsequently named ovine trophoblast protein-1 (oTP-1; Godkin et al., 1984a), was shown to consist of three similar species based on their isoelectric points between 5.3 and 5.7, each with molecular weights of approximately 17,000 (Godkin et al., 1982). The predominant secretion of oTP-1 occurred transiently between days 13 and 23, after which it was no longer detected. Trophoblast tissue, but not yolk sac, was capable of secreting oTP-1, although detectable levels of this protein within the trophoblast tissue itself were very low. The trophoblastic origin of conceptus-derived proteins was also supported by the fact that transfer of trophoblastic vesicles, obtained from day 13 ovine blastocysts from which the embryonic disk cells were removed, resulted in luteal maintenance for 20 to 54 days in nonpregnant recipient ewes (Heyman et al., 1984). Godkin et al. (1982) were able to purify oTP-1 from the culture media by successive ion exchange and gel chromatography which
provided a means of obtaining sufficient amounts of this valuable protein to continue their elegant studies.

The ability of the proteins secreted by day 15 to 16 conceptuses to extend CL life span was tested (Godkin et al., 1984b). Daily infusion of 2.2 mg of total protein recovered from media or 0.2 mg of oTP-1 into the uterine lumen of cycling ewes between days 12 and 18 post-estrus maintained luteal function. All ewes receiving total conceptus protein had functional CL out to day 25, with one ewe actively secreting progesterone until day 52. Ewes receiving oTP-1 maintained their CL four days longer than control ewes receiving diluted sheep serum. Reasons suggested for the lesser effectiveness of oTP-1 relative to total conceptus protein in prolonging CL function were that it was infused at too low a concentration, it may have been partially denatured during purification or proteolytically altered within the uterus (Godkin et al., 1984b). Alternatively, the presence of other lesser characterized proteins necessary for maternal recognition of pregnancy may be required.

The mechanism whereby oTP-1 exerts its effects appears to be of an antiluteolytic nature at the level of the uterine endometrium. Studies concerning the early hypothesis that the conceptus reduces the synthesis and/or secretion of PGF\(_2\alpha\) have yielded conflicting results. Some reports indicated that uteroovarian levels of PGF were lower during early pregnancy than during luteolysis.
In contrast, levels of PGF₂₅ in the uteroovarian veins of pregnant ewes on days 13 to 17 were reported in at least a dozen studies to be greater or not different than those in nonpregnant ewes on similar days of the estrous cycle (reviewed by Inskeep and Murdoch, 1980). Recently, both McCracken et al. (1984) and Zarco et al. (1984) provided evidence to indicate that the pulsatile release of PGF₂₅ observed at a frequency of 5 to 6 pulses per day between days 15 and 17 of the estrous cycle is reduced to only a single episode in pregnant ewes. Using a specific antiserum, Godkin et al. (1984b) localized the presence of oTP-1 to the trophectoderm cells of the blastocyst as well as the surface and glandular epithelium of the maternal endometrium. Endometrial homogenates contained the presence of high affinity receptors for oTP-1. Infusion of radioiodinated oTP-1 into the uterine lumen of nonpregnant ewes on day 12 of the cycle revealed that most of the label was retained in the uterus and did not appear to enter the maternal vasculature because no association with CL, non-luteal ovarian tissues or peripheral tissue was observed (Godkin et al., 1984b). Ovine TP-1 failed to displace both PRL and hCG from their respective receptors, nor did it stimulate progesterone production from dispersed ovine luteal cells (Godkin et al., 1984b).

These data support the suggestion that oTP-1 acts on the maternal endometrium to prevent luteolysis.
Fincher et al. (1986) provided some critical evidence to indicate the antiluteolytic properties of ovine conceptus proteins. These researchers demonstrated that daily uterine infusions of conceptus secretory proteins, obtained from day 16 blastocysts cultured in vitro, into nonpregnant ewes treated with either estradiol or oxytocin suppressed the total quantity, amplitude and frequency of pulsatile PGF₂α release. These observations support data from previous studies that indicated estradiol- and oxytocin-induced PGF₂α release in ewes is attenuated when a conceptus is present (Kittock and Britt, 1977; Fairclough et al., 1984). Conceptus secretory proteins could act to reduce PGF₂α synthesis by decreasing the concentrations of, or by binding directly to, the endometrial oxytocin receptor. The levels of oxytocin receptors observed during early pregnancy were lower than those observed during luteolysis (McCracken et al., 1984). Alternatively, conceptus secretory proteins could control luteolysis by altering enzymatic pathways that would favor an increased secretory ratio of PGE₂:PGF₂α as has been observed during early pregnancy (Silvia et al., 1984b). These provocative possibilities require further investigation.

Although the available evidence indicate the effectiveness of oTP-1 in preventing luteolysis through an interaction at the maternal endometrium, there is corroboration for the role of additional conceptus secretory
proteins as well. As the conceptus continued to develop past day 14, many additional proteins were secreted into the media (Godkin et al., 1984a), one of which was a high molecular weight (> 660,000) glycoprotein consisting of 50% carbohydrate represented by galactose, N-acetylglucosamine, mannose and fucose (Masters et al., 1982). The function of this particular protein is presently unknown, but it could be involved in the inhibition of luteolysis, protection of the conceptus from immunological attack, or cellular interaction necessary for blastocyst elongation. In addition, incubation of endometrial explants with purified oTP-1 revealed the synthesis of six polypeptides (Godkin et al., 1984b). These oTP-1-induced proteins have not been characterized to date, but it is conceivable they may act at the level of the endometrium and/or CL to participate in the maternal recognition of pregnancy. Hanson et al. (1985) have successfully isolated oTP-1 messenger RNA from day 16 conceptuses. This study indicates the exciting prospect of cloning the oTP-1 gene, and possibly those of the other proteins, which could enable the production of copious quantities of conceptus secretory proteins to further delineate their important role in regulating luteal life span.

Detectable levels of placental lactogen (oPL) are present in the ovine trophoblast as early as day 14 of pregnancy (Martal and Djiane, 1977). Ovine PL is distinct from oTP-1 in that it has a molecular weight of 22,000 and
an isoelectric point between 7.7 and 8.4 (Chan et al., 1976). This protein is most noted for its mammotrophic and somatotropic properties (Chan et al., 1976) during gestation. Specific binding of oPL to ovine CL was demonstrated (Chan et al., 1978), but its possible role in luteal function had not been tested until recently. Infusion of oPL alone or in combination with PGF₁α into the autotransplanted ovary in ewes on day 12 of an induced estrous cycle did not stimulate progesterone secretion nor prevent PGF₁α-induced luteal regression (Schramm et al., 1984). The effectiveness of oPL as an inhibitory factor on the secretion of PGF₁α by the endometrium has not been investigated. Thus, a role of oPL in the maternal recognition of pregnancy remains equivocal.

The exquisite experiments of Mapletoft et al. (1976) support the concept of local regulation of luteal maintenance by the ovine conceptus at the ovarian level. Using pregnant ewes with bilateral CL and isolated uterine horns, these researchers performed surgical anastomoses of the uterine and ovarian vasculature in different combinations to test the hypothesis that CL maintenance results from the transfer of conceptus-produced substances from the gravid uterine horn to the CL in the adjacent ovary through a venoarterial pathway similar to that described for PGF₁α. They made four major observations. First, when the main uterine vein from the gravid side was anastomosed to the main uterine vein on the nongravid
side, the CL on the nongravid side was maintained. Secondly, when the ovarian artery from the gravid side was anastomosed to the ovarian artery on the nongravid side, the CL on the nongravid side was maintained. These results support the local transfer of conceptus substances from the uterine vein to the ovarian artery to cause luteal maintenance. Thirdly, when the ovarian artery from the nongravid side was anastomosed to the ovarian artery on the gravid side at a point where no apposition occurred between the ovarian artery and uterine vein of the gravid side, the CL on the gravid side regressed. This was due to the delivery of only PGF$_2\alpha$ from the nongravid side to the CL on the gravid side. Lastly, when the ovarian artery from the nongravid side was anastomosed to the ovarian artery on the gravid side at a point where conceptus products were transferred from the uterine vein to that ovarian artery, the CL on the gravid side was maintained. These results indicate that the conceptus does not prevent luteolysis by inhibiting venoarterial transfer of PGF$_2\alpha$ since it was effective in maintaining the CL even if the ovarian artery already contained PGF$_2\alpha$. In addition, when PGF$_2\alpha$ from the nongravid horn was present simultaneously with conceptus substances in the ovarian vein ipsilateral to the gravid horn, the CL was maintained, supporting an action at the level of the ovary. The conceptus signals reaching the CL could act as antiluteolyisins or luteotropins. This countercurrent
transfer mechanism favors the notion that the conceptus signal(s) is of a small molecular weight and/or lipid-soluble such that passage through the vessel walls can be achieved. This signal has been proposed to be PGE₂.

The presence of PGE₂ in the uterine endometrium during early pregnancy in ewes was initially shown by Wilson et al. (1972). Subsequent investigations using a variety of experimental protocols delineated the effectiveness of PGE₂ in maintaining luteal function. Intrauterine infusion of PGE₂ beginning on day 12 post-estrus prolonged luteal maintenance until day 18 (Pratt et al., 1977). Estradiol-induced luteolysis was also prevented in ewes by the intrauterine administration of PGE₂ (Colcord et al., 1978). This effect of PGE₂ was exerted only when infused into the lumen of the uterine horn adjacent to the ovary bearing the CL (Magness et al., 1981). The concomitant administration of PGE₂ prevented and/or delayed luteal regression induced by arterial infusions or perivascular injections of PGF₂α (Henderson et al., 1977; Mapletoft et al., 1977; Reynolds et al., 1981).

Endometrial tissue from pregnant ewes incubated in vitro released more PGE₂ as early as day 12 than that from nonpregnant ewes (Ellinwood et al., 1979b; Marcus, 1981; LaCroix and Kann, 1982). Uterine flushing obtained from pregnant ewes also had greater concentrations of PGE₂ on day 13 than flushings obtained from nonpregnant ewes (Ellinwood et al., 1979b). Evaluation of single blood
samples collected from the uterine vein on days 15 to 17 of the estrous cycle or pregnancy either revealed no differences (Lewis et al., 1978) or increased levels during early pregnancy (Ellinwood et al., 1979b). However, a more intensive sampling regimen has shown that PGE₂ levels in uteroovarian venous blood were greater on day 13 of pregnancy than of the estrous cycle (Silvia et al., 1984b). Although uteroovarian levels of both PGE₂ and PGF₂α increased as pregnancy and the estrous cycle advanced to day 14, the PGE₂:PGF₂α ratio was maximal on day 13 of pregnancy (Silvia et al., 1984b). Ovarian arterial PGE₂ levels have not been investigated.

A contribution to PGE₂ production by the ovine conceptus is also apparent on days 14 to 16 of pregnancy (Hyland et al., 1982; LaCroix and Kann, 1982; Lewis and Waterman, 1985). These reports also showed that the ovine conceptus released more PGE₂ and PGF₂α on a per milligram of tissue basis than did endometrial tissue. However, the amount of PGE₂ synthesized by the conceptus is small when one considers this in relation to the surface area of the endometrium. Thus, it seems unlikely that the increased levels of PGE₂ observed on day 13 of pregnancy are solely attributable to the conceptus. Rather, conceptus secretory proteins may play a role in enhancing metabolic conversions of endometrial prostaglandins in favor of PGE₂.

The precise mechanism(s) whereby PGE₂ maintains
luteal function remain speculative. It is conceivable that PGE₂ may exert an antiluteolytic effect by inhibiting the ovarian vasoconstrictor effects of PGF₃α on the ovarian vasculature, as it is known that PGE₂ is a potent vasodilator (Strong and Bohr, 1967). In addition, antiluteolytic properties of PGE₂ may be manifested at the level of the luteal cell. Ovine and bovine CL contain specific binding sites for PGE₂ and PGF₃α (Powell et al., 1974; Lin and Rao, 1977; Fitz et al., 1982), yet it is unclear whether PGE₂ and PGF₃α interact in a competitive manner with the same class of receptor or with distinct classes of receptors. Alternatively, PGE₂ may be acting as a steroidogenic agent since it has been shown to increase progesterone synthesis by bovine (Marsh, 1971) and ovine (Silvia et al., 1984a) CL in vitro. Fletcher and Niswender (1982) demonstrated that PGF₃α decreased the LH-induced adenylate cyclase activity necessary for progesterone synthesis by ovine CL in vitro, but was without effect on adenylate cyclase activity regulated by PGE₂. These data suggest that PGE₂ and PGF₃α regulation of ovine luteal progesterone production occurs by separate mechanisms supporting the concept of distinct receptors, and that PGE₂ is not acting in a luteotropic manner because it is able to stimulate a pool of adenylate cyclase distinct from that regulated by LH. This concept will be discussed further in the final chapter of this review. Clearly, the functional mechanism(s) elicited by
PGE\textsubscript{2} during the maternal recognition of pregnancy in the ewe are in dire need of investigation.

A transient increase in blood flow to the gravid uterus occurs during maternal recognition in pregnancy in ewes, and was postulated to be estrogen-induced (Reynolds et al., 1984). Although synthesis of estrogens by ovine conceptuses during early pregnancy was not demonstrated (Gadsby et al., 1980), the level of estradiol-17\(\beta\) in uterine flushings from pregnant ewes was greater than that of nonpregnant ewes (Reynolds et al., 1984). Ovarian blood flow during maternal recognition has not been monitored; however, estradiol-17\(\beta\) can increase ovarian blood flow in ewes (see Ford et al., 1982). Even though the source of estrogen during early pregnancy remains equivocal, vasodilation of the uterine and/or ovarian vascular beds may enhance the transport of conceptus proteins and PGE\textsubscript{2} from the gravid uterus to the CL.

The maternal recognition of pregnancy may also involve mechanisms that prevent rejection of the fetus as an allograft due to its complement of paternal genes (Beer and Sio, 1982). Some of these may include the masking of surface antigens on trophoblast cells by trophoblast- and/or maternally-derived agents. In the ewe, a substance called "early pregnancy factor" (EPF; Nancarrow et al., 1981) is present in the maternal circulation and many tissues including the CL from the time of fertilization. Removal of the conceptus between days 19 and 21 caused a
concomitant decline in the ability of EPF to be detected in the maternal circulation. Proteins derived from ovine uterine fluid collected on day 14 of pregnancy suppressed T-lymphocyte proliferation in vitro (Segerson, 1981) and appears to occur at the level of the uterus (Segerson and Libby, 1984). It is possible that the uncharacterized conceptus secretory proteins may possess immunosuppressive properties that are necessary for the maternal recognition of pregnancy.

In summary, the critical period for the establishment of pregnancy as indicated by the prolongation of luteal life span in the presence of the conceptus is characterized by many events. Maintenance of luteal function appears to require a combination of antiluteolytic and steroidogenic mechanisms elicited by conceptus secretory proteins and PGE2 of both conceptus and endometrial origin. Possible roles of estradiol and pregnancy-specific antigens are evident, but not yet clearly defined. However, it is very clear that the aforementioned conceptus signals cannot substitute for the absolute requirement of pituitary LH in maintaining CL function during the maternal recognition of pregnancy in the ewe.
Mechanism of Action of Luteinizing Hormone in the Corpus Luteum

Paramount to the elucidation of LH action were two early observations: 1) the concentration of LH in the ovarian venous blood as measured by radioimmunoassay was less than that in systemic venous blood (Naftolin et al., 1968) and 2) isotopically labeled human chorionic gonadotropin (hCG) was differentially accumulated and retained in rat ovaries (Espeland et al., 1968). The first reports to document the nature of gonadotropin retention by ovarian cells was provided by Lee and Ryan (1971, 1972), who demonstrated that labeled hLH bound to slices of pseudopregnant rat ovaries with high affinity and could only be displaced by molecules containing LH activity. Thus, the concept of hormone "receptors" for LH was initiated, and provided the framework for numerous investigations concerning the binding of LH and hCG to ovarian receptors in a large number of species.

Luteinizing hormone of anterior pituitary origin and hCG of placental origin are both dimeric polypeptides, consisting of two dissimilar subunits, designated α and β, which are associated noncovalently. The α subunits of the two gonadotropins are identical, and the β subunits, which are responsible for conferring biological specificity among all the glycoprotein hormones, are closely related with an amino acid sequence homology of 82% (for review,
see Pierce and Parsons, 1981). A structural comparison of ovine LH (oLH) and hCG is presented in Table 2. Although there are relatively few physical differences between these two gonadotropins, the most notable are 1) the higher carbohydrate content of hCG, particularly that of sialic acid, which is known to contribute to its longer half-life in the circulation, and 2) an additional 28 amino acids on the C-terminus of hCG. The α subunit of LH and hCG is encoded by a single gene expressed in the pituitary and placenta, respectively. The β subunit of LH is also represented by a single gene, while β hCG is encoded by six nonallelic genes, only three of which are expressed (Fiddes and Talmadge, 1984; Policastro et al, 1986). It is believed that LH β and hCG β have evolved from a common ancestral coding sequence.

Due to their structural and functional similarities, LH and hCG bind to the same receptor site in gonadal tissues, therefore the receptor will be referred to as the "LH receptor" throughout this discussion. The LH receptor has been localized predominantly in the plasma membrane of luteal cells as indicated by subcellular fractionation (Gospodarowicz, 1973a; Menon and Kiburz, 1974; Bramley and Ryan, 1978), autoradiographic (Anderson et al., 1979) and ferritin conjugate localization (Luborsky et al., 1979) studies. The plasma membrane is the initial site of action of LH in triggering a cascade of biological events resulting ultimately in the expression of a functional
Table 2. Structural properties of oLH and hCG.

<table>
<thead>
<tr>
<th>Property</th>
<th>oLH</th>
<th>hCG</th>
</tr>
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<tbody>
<tr>
<td>Mr</td>
<td>28-33,000</td>
<td>38-48,000</td>
</tr>
<tr>
<td>% carbohydrate</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>mannose</td>
<td>7.2</td>
<td>11.4</td>
</tr>
<tr>
<td>galactose</td>
<td>1.1</td>
<td>12.1</td>
</tr>
<tr>
<td>fucose</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>GluNAc</td>
<td>8.5</td>
<td>16.4</td>
</tr>
<tr>
<td>GalNAc</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td>sialic acid</td>
<td>0</td>
<td>10.9</td>
</tr>
<tr>
<td>plasma t1/2</td>
<td>0.5 hr</td>
<td>4.9 hr</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>α</th>
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<tbody>
<tr>
<td>no. amino acids</td>
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<td>119</td>
<td>92</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>no. carbohydrate units</td>
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<td>1</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>glycosidic linkage</td>
<td>N-asn</td>
<td>N-asn</td>
<td>N-asn</td>
<td>N-asn, O-ser</td>
<td></td>
</tr>
<tr>
<td>no. S-S</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
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</tr>
</tbody>
</table>

\(^{a}\) Taken from Pierce and Parsons (1981) and Sairam (1983).
corpus luteum. The mechanism of action of LH is generally accepted to follow the "second messenger" model originally proposed by Sutherland (Haynes et al., 1960) and reviewed for corpora lutea by Marsh (1976). Briefly, LH binds to its receptor in the plasma membrane of the luteal cell wherein adenylate cyclase is activated to convert adenosine triphosphate (ATP) to adenosine-3',5'-cyclic monophosphate (cAMP), the second messenger, which in turn activates cAMP-dependent protein kinase(s). Protein kinase activity results in the phosphorylation of steroidogenic enzymes and/or other proteins necessary for the synthesis and secretion of progesterone. It is within this general framework that the mechanism of action of LH will be discussed.

Structural Properties of the LH Receptor

Purification of the LH receptor has been difficult due to its lability during solubilization from target organs resulting in low yields of receptor, its low concentration in these tissues, and the lack of large quantities of tissue necessary for detailed characterization studies. The improvement of technique has greatly aided in the recovery and stabilization of the receptor during purification. Only during the past three years has successful documentation regarding LH receptor structure been available. Unfortunately, there have been no reports to date concerning the structural characteristics of LH
receptors purified from ovine corpora lutea. Therefore, the following discussion will concern information obtained from ovaries of other mammalian species.

The purification schemes employed by the various investigators to isolate relatively pure, homogenous and stable preparations of LH receptors can be generally summarized as follows: crude or purified ovarian (luteal) plasma membranes are first detergent-solubilized and then subjected to gel filtration and/or density gradient centrifugation. Further analysis of LH receptor subunit structure is achieved following immunoaffinity chromatography, direct affinity chromatography, chemical covalent cross-linking of the gonadotropin (either LH or hCG) to the receptor using bifunctional reagents or derivatization of the gonadotropin with a photoaffinity reagent followed by radiiodination of this derivative. Purifications are carried out under nonreducing conditions, or in the presence of reducing agents (2-mercaptoethanol) to assess the possible contribution of interpeptide chain disulfide bonds.

The detergent-solubilized rat ovarian receptor linked to radiiodinated hCG isolated under non-reducing conditions by Hwang and Menon (1984) revealed a single band corresponding to a molecular weight of 305,000. When subjected to reducing agents, the appearance of four bands of Mr = 105,000, 96,000, 74,000 and 62,000 were observed, both in the presence and absence of protease inhibitors,
concomitant with the loss of the 305,000 MW band. These researchers concluded that the LH receptor is an oligo-
meric complex linked by disulfide bonds that can be
dissociated into four nonidentical subunits by reducing
agents, but not by proteases. Using a photoaffinity
labeled hCG preparation, Rapoport et al. (1984) isolated
the rat ovarian LH receptor under reducing conditions
without protease inhibitors and reported three components
with $M_r = 106,000$, 85,000 and 80,000. However, only the
largest component was able to bind hCG with high affinity
in a saturable manner, thus representing the receptor as
$M_r = 86,000$ when the MW of the covalently-linked $\alpha$ subunit
of hCG is subtracted. Similarly, Metsikko (1984) observed
a single $M_r = 90,000$ sialoglycopolypeptide with a sedi-
mentation coefficient of 5.1 S, but this component was
biologically inactive with respect to hormone binding
after purification. Recent analysis of cross-linked
gonadotropin-receptor complexes from rat ovaries under
nonreducing conditions indicated that the molecular weight
of the intact receptor is 268,000 (Bruch et al., 1986).
Under reducing conditions and in the presence of protease
inhibitors, intact receptor was dissociated into four
nonidentical polypeptides of $M_r = 79,300$, 66,400, 55,300
and 46,700 which is in close agreement with that reported

Porcine granulosa cell LH receptors were cross-linked
to either the $\alpha$ or $\beta$ subunit of a photoaffinity labeled
preparation of hCG in the presence of protease inhibitors (Ji and Ji, 1981). When the β subunit was labeled, three components of 106,800, 88,000 and 83,000 were observed; with the label in the α subunit, four components of 120,000 (occurred inconsistently), 96,000, 76,000 and 73,000 were noted. Subtracting the molecular weights of each subunit from its respective receptor components then yielded receptor subunits with $M_r = 81,000, 63,000$ and 58,000. The estimated molecular weight of the holo-receptor was found to be $250,000 - 370,000$, and reducing agents had no effect on the formation of the three components (Ji et al., 1981). More recent studies from this same laboratory (Shin et al., 1986) subjected the three receptor components obtained under reducing conditions to further reduction. They observed the formation of a 24,000 component that appears to be the initial site for photoaffinity labeling that is disulfide linked to a 28,000 component and that is in turn linked to a 34,000 component. In addition, there was no evidence of disulfide linkages between the hormone and any of these reduced receptor components. Purified LH receptors obtained under nondenaturing conditions from porcine CL (Wimalesena et al., 1986) revealed three species of $M_r = 260,000, 130,000$ and 60,000 as identified by specific binding of labeled hCG. In order to increase accuracy of detection, purified receptors themselves were radioiodinated, and migrated to a $M_r = 60,000$. Under nonreducing conditions,
identical components of this porcine LH receptor were observed. Wimalasena et al. (1986) suggest that the luteal porcine LH receptor may consist of a $M_r = 60,000$ monomer that can associate to form polymeric receptor complexes.

One of the most comprehensive studies of the LH receptor has been conducted by subjecting large quantities of bovine corpora lutea, 1200 ovaries obtained biweekly, to purification procedures (Dattatreyamurty et al., 1983) resulting in high yield, purity and stability. The bovine luteal LH receptor was found to exist as a $M_r = 280,000$ species which is composed of two identical $M_r = 85,000$ and 38,000 species. The smallest subunits appear to be linked by disulfide bonds. The amino acid composition of the purified receptor revealed a low cysteine content that distinguished it from bovine LH, and a predominance of glutamic and aspartic acid residues. In addition, the receptor was found to consist of 10% carbohydrates which included mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid. The rat ovarian LH receptor has also been found to be a glycoprotein (Metsikko, 1984; Bruch et al., 1986).

In summary, the conflicting observations that led to the proposed structure of the LH receptor can be divided into two groups: 1) the LH receptor is a noncovalently bonded oligomer of a single polypeptide or a small polypeptide which in turn is composed of two disulfide-bonded
polypeptides; and 2) the LH receptor is an oligomer consisting of four nonidentical subunits joined either by noncovalent or disulfide bonds. There appears to be some consensus on the structure of the LH receptor containing carbohydrates. The reasons for the discrepancies reported in the subunit structure and their heterogeneous Mr values remain to be resolved and may involve the purification techniques employed, failure to inhibit protease activity, inaccurate Mr determination due to the carbohydrate portion of the receptor and/or actual species differences. Another important issue that remains to be addressed is the valency of the holoreceptor and its various subunits; it is not known whether the purified receptor is monovalent, wherein each subunit binds to a different site on the hormone, or multivalent, with each subunit capable of binding to the same site on the hormone. Purification of the LH receptor will continue to be an intense area of investigation that should aid in the resolution of the currently conflicting evidence.

LH Receptor Binding Affinity and Kinetics

Considerable data exist defining the structural regions of the LH/hCG that are involved directly with receptor recognition, and have been described in detail by Pierce and Parsons (1981) and Sairam (1983). Hormonal activity is only expressed after interaction of both the α and β subunits with the receptor. The receptor binding
domains of the α subunit of LH include the carboxy terminal pentapeptide, lys-55, met-51 and the disulfide linkage between cys.11-35, while the lys-94 and met-41 of the β subunit are also required. Based on a number of studies involving gonadotropin structural modifications, a minimum of three receptor recognition domains, two contributed by the α subunit and the third by the β subunit, is necessary for receptor binding. A "determinant loop" hypothesis (see Sairam, 1983) has also been proposed to explain the hormonal specificity conferred by glycoprotein hormone β subunits with respect to receptor binding. According to this hypothesis, the nature of the octapeptide loop amino acid composition formed by the disulfide bridge between cys-93 and cys-100 of the β subunit determines whether the α-β complex will recognize an LH, FSH or thyroid stimulating hormone (TSH) receptor. It is presumed that the loop is located on the surface in the intact α-β complex and functions to direct specific binding to the receptor. The loop contains nonconservative amino acid substitutions that contribute to the overall net electrostatic charge in this region. Either a neutral or net positive charge in the loop is required for oLH/hCG biological activity, whereas a net negative charge confers FSH and TSH activity that are distinguished by the absence of a tyr in the FSH loop. It is possible there are other domains in the three dimensional structure of LH/hCG that contribute to receptor recognition.
The complex oligosaccharide moieties present in LH/hCG appear to be nonessential for receptor binding. Deglycosylated (DG) derivatives of LH/hCG including DG-native hormone and recombinants of DGα-β, α-DGβ and DGα-DGβ are all capable of binding to the LH receptor (Sairam, 1983). In fact, deglycosylation has been shown to enhance receptor binding mainly by increasing the "on-rate", or rate of association, of LH with its receptor (Liu et al., 1984). It must be emphasized, however, that the carbohydrate composition of the α subunit is an absolute requirement for the stimulation of cellular events that occur post-receptor binding (Sairam, 1983). Deglycosylation of the native LH/hCG and the DGα-β, DGα-DGβ recombinants exhibits attenuated or total lack of adenylate cyclase stimulation and steroidogenesis. The α-DGβ recombinant, however, is nearly equipotent as native LH/hCG in stimulating cyclase and steroid production. The precise structural location and composition of the carbohydrate moieties in the α subunit necessary for biological activity are unknown.

Equilibrium binding data are routinely derived from saturation analysis or binding-inhibition assays (Catt et al., 1976), and analyzed by applying the Scatchard (1949) model. These methods quantify only those receptors unoccupied by hormone. Luteal tissue has been found to contain a single population of LH receptors exhibiting equilibrium dissociation constants (Kd×10^{-10} M) of 0.87 in
ovine (Diekman et al., 1978a), 0.07-0.28 in bovine (Haour and Saxena, 1974; Spicer et al., 1981), 0.57-3.0 in porcine (Ziecik et al., 1980; Bramley, 1981) and 0.60 in primate (Cameron and Stouffer, 1982a) luteal plasma membrane. It is difficult to compare the total numbers of unoccupied receptors present in luteal tissues because of the variation in the reference base used (mass of CL wet weight, protein, DNA or cell number), but they have been reported to range from 10,000-30,000 sites per cell in bovine (Papaioannou and Gospodarowicz, 1975) and ovine (Fitz et al., 1982) corpora lutea.

The association of LH to its receptor is independent of both the concentration of receptor and hormone (Lee and Ryan, 1973); thus it follows second-order kinetics, with calculated association rate constants ($k_a$) ranging from $1.8-3.0 \times 10^6 \text{ m}^{-1} \text{ sec}^{-1}$ at or above room temperature in bovine (Haour and Saxena, 1974; Ahzar and Menon, 1976) and primate (Cameron and Stouffer, 1982a) luteal plasma membranes. The $k_a$ is quite temperature-dependent, and has been shown by numerous investigators to be decreased particularly at 4 C. The dissociation of LH from luteal membrane preparations at room temperature is a biphasic reaction indicating a fast and a slow phase. The calculated dissociation rate constants ($k_d$) are $0.6-0.7 \times 10^{-3} \text{ m}^{-1} \text{ sec}^{-1}$ and $6 \times 10^{-6} \text{ m}^{-1} \text{ sec}^{-1}$ for the fast and slow pools, respectively, in bovine (Gospodarowicz, 1973b) and primate (Cameron and Stouffer, 1982a) luteal
membranes. For example, only 40% of labeled hCG was
dissociated from primate luteal membranes within 8 hours
at room temperature, with virtually no further dissociation
for up to 20 hours (Cameron and Stouffer, 1982a). At
4 °C, no dissociation occurs over 140 hours (Lee and Ryan,
1973). These data may seem to indicate that the dissociation
is an irreversible reaction involving covalent
binding of the hormone to the receptor. This is not the
case, however, because nearly all of the bound LH/hCG can
be dissociated when luteal membranes are exposed to
buffers of acidic pH or high ionic strength MgCl₂ (Diekman
et al., 1978a; Cameron and Stouffer, 1982a) apparently
without compromising the immunological and biological
activity of the hormone. In fact, stripping the hormone
from the receptor using these methods with subsequent
radioimmunoassay (RIA) of hormone concentration is
routinely employed in the measurement of occupied recep-
tors for a number of polypeptide hormones. Thus, the
binding of LH/hCG does not in fact conform to the simple,
bimolecular reversible reaction implicitly assumed when
using Scatchard analysis, and suggests that an additional
model of binding can account for the linearity of the
Scatchard plots indicative of a single class of receptors.
The elegant treatise by Moyle (1980) explains that linear
Scatchard plots can be obtained using a model where the
initial hormone-receptor complex, representing the pool of
receptors capable of rapid hormone dissociation, undergoes
further modification to form a hormone-receptor complex reflecting the slowly dissociating pool. This latter form of the hormone-receptor complex has been suggested to possibly represent those that are involved with the activation of adenylate cyclase and/or undergoing processing, such as internalization. In summary, the binding kinetics of LH/hCG may simultaneously represent multiple hormone-receptor interactions.

LH Receptor Concentrations during the Estrous Cycle and Pregnancy

Because it has been known for more than a decade that LH is necessary for maintaining luteal progesterone synthesis in domestic animals and that the initial step in the mechanism of action involves interaction with a membrane receptor, the obvious sequelae of investigations conducted by animal scientists involved the quantification of LH receptor concentrations during the estrous cycle and the correlation with progesterone production. These studies provided an important basis for investigating luteal function in species with comparatively longer estrous or menstrual cycles than those of laboratory animals.

Diekman et al. (1978a) quantified the numbers of both occupied and unoccupied luteal LH receptors in crude plasma membrane preparations obtained from ewes on days 2 through 16 of the estrous cycle. The number of occupied
LH receptors increased 84% from minimal levels on day 2 to maximal levels on day 10, remained elevated through day 14, and then declined by 75% on day 16. The levels of unoccupied LH receptors exhibited parallel changes, and were highly correlated \((r^2 = .704)\) to occupied receptor concentrations. The total number of receptors increased 40-fold during development of the functional CL. Similarly, CL weight, serum and luteal progesterone concentrations were also significantly correlated \((r^2 = .715- .785)\) with occupied LH receptor levels. The increase in receptor numbers was not associated with changes in the affinity of the LH receptor on any day of the estrous cycle. Diekman et al. (1978a) suggested that the increased number of LH receptors appeared to be the major factor in the regulation of luteal progesterone secretion. A direct cause and effect relationship between the decline in luteal LH receptors and progesterone secretion on day 16 may not be valid in view of the fact that serum levels of progesterone declined 14.5 hours earlier than the number of occupied and unoccupied LH receptors in day 9 CL from PGF\(_2\alpha\)-treated ewes (Diekman et al., 1978b). Therefore, while increased LH receptors may appear to regulate luteal progesterone synthesis, a decrease in LH receptors is not the primary locus of action determining the reduction in luteal function at the end of the estrous cycle.

Maximal binding of hCG during the mid-luteal phase of the estrous cycle was also shown to occur in bovine
(Spicer et al., 1981), equine (Roser and Evans, 1983) and porcine CL (Ziecik et al., 1980). The correlation between LH receptor number and CL function is also expressed during the human (Rao et al., 1977) and primate (Cameron and Stouffer, 1982b) menstrual cycles.

Diekman et al. (1978a) also investigated the possibility that maintenance of the ovine corpus luteum during early pregnancy involved changes in the number and/or affinity of luteal LH receptors. These researchers observed that the number of unoccupied and occupied LH receptors in CL obtained from ewes on days 12, 16 and 20 of pregnancy were identical to those quantified on day 12 of the estrous cycle. Thus, the presence of the embryo is affecting some other process(es) involved in luteal maintenance.

LH Receptor Occupancy and Response

Two of the earliest investigations into the relationship between receptor occupancy and biological responses were conducted by Catt and Dufau (1973) and Mendelson et al. (1975). Using isolated rat Leydig cells exposed in vitro to hCG, these researchers showed that only 1-2% of the LH receptors needed to be occupied to elicit maximal stimulation of testosterone synthesis, while 40-50% occupancy was necessary for maximal stimulation of cAMP production. Under physiological conditions in the ewe during the mid-luteal phase of the estrous cycle and
early pregnancy, Diekman et al. (1978a) indicated that only 0.6% of the total luteal LH receptors were occupied when progesterone production was maximal. Thus, an apparent discrepancy exists between receptor binding and biological response because there are far more receptors present in gonadal tissue than are necessary to invoke maximal steroidogenesis. The spare receptor concept (Catt and Dufau, 1973) has been forwarded as an explanation for this paradox, wherein only a small proportion of receptors are coupled to the response mechanism. Although the precise physiological function of spare receptors is unknown, Moyle (1980) has reviewed some possible explanations for their existence. First, a large number of spare receptors may be necessary to increase the sensitivity of gonadal tissue to low concentrations of circulating gonadotropins. This may be particularly significant during the mid-luteal phase of the estrous cycle and early pregnancy in ewes when maximal progesterone synthesis occurs and when serum LH levels are at their nadir (Niswender et al., 1968). The presence of spare receptors would thus preclude the need for increased hormone secretion rates. Secondly, the presence of spare receptors may serve to amplify small physiological fluctuations in plasma gonadotropin levels as indicated by increases in the slope of dose-response curves obtained experimentally. In the absence of spare receptors, a 100-fold increase in plasma hormone levels would be needed to increase the
response from 10 to 90% of maximum. In contrast, in the presence of 100-fold spare receptors, only a 9-fold change in hormone levels would be adequate to raise the response. Lastly, spare receptors may be needed for the occurrence of a quantal (all-or-none) response (Moyle et al., 1985), wherein a change in the threshold number of receptors occupied by hormone would lead to variations in cellular response in the absence of changing plasma hormone concentrations. For example, 0.1% occupancy of rat Leydig cells by hCG is insufficient for induction of testosterone synthesis, but in the presence of phosphodiesterase inhibitors this low degree of occupancy could now elicit a response. Moyle et al. (1985) suggested that the Leydig cell has a threshold number of receptors that must be exceeded for a response to occur, and that the size of the threshold is independent of receptor occupancy, but could be dependent upon intracellular levels of cAMP that would dictate the threshold degree of receptor occupancy needed for a biological response. This would be an interesting aspect to pursue in the two different cell populations of the ovine CL, and may explain why the large luteal cells are able to secrete maximal levels of progesterone in vitro without the addition of exogenous LH. One may speculate that possibly the isolated large cells, which could retain a small proportion of LH bound to their receptors, have lower levels of phosphodiesterase activity resulting in higher levels of intracellular cAMP that
would preclude the necessity for a large receptor occupancy necessary to induce progesterone synthesis. The degree of receptor occupancy and possible involvement of the threshold model in the contribution of small and large ovine luteal cells to overall progesterone production during physiological conditions has not yet been investigated experimentally. Niswender et al. (1985a) state that large luteal cells are not under the direct control of LH, yet no studies have been conducted to determine the degree of receptor occupation in large cells post-isolation, and whether large cells stripped of endogenous LH retain their capacity to secrete progesterone at a maximal rate or require a lower than expected occupancy of LH receptors.

Homologous Regulation of Luteal LH Receptor Turnover

The previous discussion of LH receptor concentrations in functional ovine CL revealed that receptor levels were elevated, yet constant. These observations reflect only the events occurring in the population of luteal cells as a whole at a given steady-state stage in their transient life span. However, the homologous regulation of luteal LH receptors is probably more accurately explained by the dynamic processes of up- and down-regulation that occur within each individual luteal cell upon exposure to LH. The currently accepted cellular mechanisms regulating LH receptor turnover by homologous gonadotropins, LH/hCG, will be presented below.
The primary effector regulating luteal LH receptors in sheep appears to be LH. Indirect evidence for this hypothesis is indicated by 1) the loss of luteal function when the source of circulating LH is removed by hypophysectomy and restored by continuous infusion of LH (Kaltenbach et al., 1968) and 2) the ability of LH to increase the synthesis and secretion of ovine luteal progesterone in vivo (Niswender et al., 1976) and in vitro (Kaltenbach et al., 1967; Simmons et al., 1976). More direct evidence indicated that neutralization of endogenous LH by injection of anti-oLH serum into ewes on day 9 of the estrous cycle reduced CL weight, levels of unoccupied and occupied LH receptors and serum progesterone 12 hours post-injection (Niswender et al., 1981).

The changes in LH receptor levels in luteal tissue follow a somewhat characteristic time curve following exposure to LH/hCG and are dependent upon the dose and nature of the gonadotropin. Suter et al. (1980) provided interesting evidence for the temporal regulation of ovine luteal LH receptors following the exogenous administration of a supraphysiological dose (1 mg) of oLH to ewes on day 10 of the estrous cycle. Within 10 minutes of the injection, serum LH increased 1000-fold and was accompanied by a 260% increase in the total number of LH receptors that included a 13-fold increase in occupied receptor levels. A similar phenomenon was reported to occur in rat testes within one hour post-treatment with 1 or 20 µg of
hCG and 100 µg oLH (Huhtaniemi et al., 1981). This transient positive regulation of LH receptors by homologous hormone has been designated "up-regulation", and is correlated with increases in steroid synthesis. The appearance of the increased receptors has been suggested to occur via structural changes and/or increased fluidity of gonadal plasma membranes that would lead to exposure of cryptic or masked surface receptors (Huhtaniemi et al., 1981). In support of this hypothesis, Danforth et al. (1985) recently demonstrated that in vitro treatment of primate luteal crude plasma membrane preparations with ethanol resulted in increased membrane fluidity highly correlated with an increase in LH receptors believed to represent masked receptors. Alternatively, the up-regulation of LH receptors could be accounted for by the insertion of recycled hormone receptors into the plasma membrane. Niswender et al. (1982) also showed that up-regulation of ovine luteal LH receptors in response to 1 mg oLH in vivo occurred in a linear fashion for up to 30 minutes post-injection and was reflected by increases in serum progesterone. Suter et al. (1980) proposed that the LH-induced exocytosis of Golgi-derived secretory granules that occurs concomitantly with progesterone secretion (Gemmell et al., 1977; Sawyer et al., 1979) may be inserting LH receptors into the plasma membrane during this process. Similarly, pretreatment of rat testes with cytochalasin B, a microfilament inhibitor, and amino-
glutethamide, a steroidogenesis inhibitor, blocked up-regulation of LH receptors (Huhtaniemi et al., 1981). Although not proven unequivocally, up-regulation may provide a mechanism to replace receptors lost from the plasma membrane.

The up-regulation of ovine luteal LH receptors in response to oLH was transient, and occupied LH receptors returned to preinjection levels by 6 hours post-injection (Suter et al., 1980) where they remained at levels similar to pre-injection receptor numbers for up to 72 hours post-treatment. The number of unoccupied LH receptors decreased between 10 minutes and 24 hours post-injection, but returned to pre-injection levels at 48 and 72 hours. Total numbers of LH receptors decreased by 63% at 24 hours post-injection when compared to pretreatment levels, but returned to control levels at 48 hours. This net loss in LH receptors after exposure to homologous hormone is known as "down-regulation", and has also been documented in luteinized rat ovaries where as little as 10 ng hCG could decrease total LH receptor levels by 90% 24 hours post-injection (Harwood et al., 1978). The total number of LH receptors lost between 12 and 24 hours after injection of oLH was in good agreement with the number of receptors occupied at 10 minutes post-injection (Suter et al., 1980), indicating a possible relationship between receptor loss and occupancy. Interestingly, the 63% decline in total ovine luteal LH receptors after the injection of LH
was not accompanied by a decline in serum progesterone, which remained at or above that observed in control ewes at all times during the study. Maintenance of progesterone secretion may have been due to the fact that occupied LH receptor levels also never decreased below levels observed prior to the injection of LH. In contrast to these data, the down-regulation of LH receptors in luteinized rat ovaries induced by exogenous hCG did result in decreases in progesterone synthesis (Harwood et al., 1978). Furthermore, levels of total LH receptors in hCG-treated rats remained depressed from 1 to 4 days post-treatment, and did not recover to pre-injection values until 4 to 7 days later. The reasons for the apparent discrepancy between the ovine and rat data are not known, but could be due to actual species differences and/or the use of relatively high doses of hCG that may have remained in the circulation of the rats for a longer period of time.

Internalization of the LH-Receptor Complex

A number of protein hormones that bind to cell surface receptors are internalized intracellularly by a mechanism referred to as receptor-mediated endocytosis (see Goldstein et al., 1980, for review). According to this model, the hormone-receptor complexes cluster on the cell surface in specialized areas called coated pits that are characterized by the presence of a transmembrane
protein identified as clathrin. The coated pits subsequently invaginate, the clathrin forms a hexagonal protein network thus surrounding the hormone-receptor complexes, and the resultant coated vesicles are internalized. The cellular fate of the internalized hormone is presumably degradation by lysosomal enzymes, while the receptor may be recycled to the plasma membrane. Other proteins may be re-directed to other subcellular organelles where they continue to express a biological function, while evidence also exists for the transfer of certain proteins through the cytoplasm to be delivered to another cell type or to the circulation. Receptor-mediated endocytosis has been generally accepted to be the major mechanism explaining down-regulation of LH receptors.

Autoradiographic and indirect immunofluorescence techniques revealed that 70% of the labeled hCG-receptor complexes were randomly distributed on the plasma membrane surface of rat granulosa cells within 2 hours, while the remaining 30% appeared to move laterally within the plane of the plasma membrane to aggregate into large clusters (Amsterdam et al., 1979). Using a ferritin-LH conjugate, Luborsky and Behrman (1979) observed a uniform distribution of the label over isolated rat luteal cell plasma membranes at 4 C, but at 37 C the cells exhibited small, variable sized patches, or "microaggregates", occurring at irregular intervals along the cell surface. In vivo administration of isotopically labeled hCG into rats
showed LH receptor-complexes to be distributed mainly on the microvilli and folded regions of luteal cell membranes adjacent to vascular spaces, with less labelling observed in the basolateral portions of plasma membranes (Han et al., 1974; Anderson et al., 1979). Similarly, Chen et al. (1977) observed that 10 to 30 minutes post-injection of labeled hCG into the ovarian artery of ewes on day 9 of the estrous cycle resulted in most of the label being localized over microvilli and "long, pleomorphic cytoplasmic folds" that extended from the main body of large luteal cells, while small luteal cells were uniformly labeled on the plasma membrane over their entire perimeters. Thus, the distribution of LH-receptor complexes subsequent to hormone binding remains equivocal and may be due to differences in technique and/or the use of isolated cells as compared to those prepared from animals after labeling luteal cells in vivo. However, agreement exists among data from all studies that over time, internalization of the labeled bound hCG occurred and was observed to be associated with cytoplasmic vesicles and lysosomes. Using a filtration technique designed to separate plasma membranes from secretory granules, Niswender et al. (1980) demonstrated that about 40% of the hCG specifically bound to ovine luteal cells could be recovered from intracellular sources. When this fraction was solubilized and subjected to centrifugation on sucrose gradients, one-fourth of the label was repre-
sented by free hCG while the remainder migrated to a position in the gradient similar to solubilized hCG-receptor complexes (Conn et al., 1978). Therefore, it appears that the LH receptor is internalized along with its bound hormone.

The studies of LH receptor distribution presented above fail to document whether aggregation of LH-receptor complexes is necessary for internalization, because it was not possible to follow the localization over time within the same cell. However, a deglycosylated preparation of hCG did not aggregate on the surface of rat luteal cells as shown by indirect immunofluorescence, yet was found to be internalized to the same extent as native hCG (Thotakura and Bahl, 1983). Based on this study, removal of the carbohydrate moieties of hCG results in a loss of receptor aggregation, but aggregate formation may not be a prerequisite for internalization. The possible biological relevance of aggregation will be discussed in later sections.

The kinetics of the internalization and degradation of receptor-bound oLH, hCG and various recombinant preparations were demonstrated in ovine CL by a technique developed by Ahmed et al. (1981). Luteal cells were labeled with hCG for 10 to 20 minutes, washed, resuspended and incubated at 37°C for various periods of time. The medium was subjected to trichloroacetic acid (TCA) treatment with the TCA-precipitable material representing degraded hCG in
the form of mono- and diiodotyrosine. The cells were treated with acetic acid and centrifuged, with the supernatant representing membrane-bound hCG and the pellet reflecting nonreleasable hormone. The quantity of internalized hCG was the sum of the degraded and nonreleasable hCG. In the initial study, hCG was lost from the plasma membrane with a $t^{1/2} = 9.6$ hours, with 85% of the initial bound hCG gone by 24 hours (Ahmed et al., 1981). In addition, these researchers also showed that internalized hCG increased to a plateau at 4 hours, remained stable until 12 hours and then decreased to 24 hours, while degraded hCG increased linearly up to 24 hours. Thus, the $t^{1/2}$ of internalization plus degradation of hCG was 17 hours, which agrees closely with the loss of labeled hCG from ovine CL in vivo (Chen et al., 1977). Similarly, 80% of labeled hCG bound to rat granulosa cells in vitro was released into the media as iodotyrosine following 24 hours of incubation (Amsterdam et al., 1979). In addition, media removed from prelabeled, washed large ovine luteal cells during 3 to 18 hours of incubation displayed a marked inability to rebind to excess LH receptors (Niswender et al., 1980), indicating hormone degradation following receptor binding.

Subsequent studies utilizing ovine CL revealed differences in the rates of internalization of various forms of LH and hCG. The $t^{1/2}$ for loss of membrane bound hormone was shown to be 22, 23, 15 and 0.4 hours for hCG,
asialo hCG, hLH and oLH, respectively (Mock and Niswender, 1983). In addition, the quantity of internalized hormone (nonreleasable plus degraded) was greater over the first two hours of incubation for oLH than the other three gonadotropins. The observation that the hCG and hLH had slower internalization rates than oLH was attributable to the greater carbohydrate content of these gonadotropins, and that sialic acid residues did not contribute to this difference. Further characterization of the portion of the gonadotropin structure that was rate-limiting for internalization indicated that, in general, recombinant preparations of LH/hCG containing the β subunit from hCG were internalized and degraded more slowly \( t^{1/2} = 9 \) to 22 hours) than those containing the β subunit from oLH \( t^{1/2} = 0.5 \) to 0.7 hours; Mock et al., 1983). In addition, when the radioiodine was incorporated into the β subunit of oLH, degradation and internalization occurred more rapidly than when the radioiodine was present in the α subunit, yet the significance of this remains unknown. Recent elegant studies by Niswender et al. (1985a) provide a plausible explanation for why receptor-bound hCG is internalized 50 times slower than receptor occupied by oLH. Using biologically active rhodamine-labeled oLH and hCG preparations bound to ovine luteal cells in vitro in the presence of sodium azide to prevent internalization and subjecting these cells to photobleaching by laser light, the times for the fluorescent hormone-receptor
complexes to diffuse into the bleached membrane region were measured. The diffusion coefficients for receptor complexes bound by oLH and deglycosylated hCG were $1.9 \times 10^{-10}$ cm$^2$/sec$^{-1}$ and $1.1 \times 10^{-11}$ cm$^2$/sec$^{-1}$, respectively, which were comparable to that of cell surface glycoproteins labeled nonspecifically with succinylated concanavalin A. The fluorescence recovery after photobleaching for the hCG-LH receptor complexes was so low, $< 1 \times 10^{-11}$ cm$^2$/sec$^{-1}$, as to imply immobilization of the receptor within the membrane. Extending these observations, Roess et al. (1986) observed that in colchicine- and cytochalasin-D-treated ovine luteal cells, as well as in luteal membrane "bleb" preparations free of underlying cytoskeletal components, rhodamine-labeled hCG-LH receptor complexes were now able to diffuse within the membrane as indicated by a similar diffusion coefficient ($1.7 \times 10^{-10}$ cm$^2$/sec$^{-1}$) obtained previously with receptor-bound oLH. Taken collectively, these data suggest that the carbohydrate portion of the β subunit of hCG may be interacting with microtubules and microfilaments in the vicinity of the plasma membrane, thus rendering the hCG-LH receptor complex immobile and thereby increasing the time it remains on the luteal cell surface before internalization occurs. It is not known from these studies whether 1) microaggregation or clustering of the hormone-receptor complexes occurred; 2) the carbohydrate moieties of hCG interact directly with the LH receptor or with the membrane-associated cytoskeletal
components; or 3) the carbohydrate portion alters the tertiary structure of the peptide portion of hCG to induce immobilization. These observations will provide the basis for some interesting future studies regarding the mechanism of LH-receptor complex internalization.

Once internalized, the gonadotropin is believed to undergo degradation in the lysosomal compartment of the luteal cell. Autoradiographic studies provide evidence for the accumulation of labeled hCG in lysosomes of ovine luteal cells (Chen et al., 1977). Treatment of ovine luteal cells in vitro with chloroquine, an inhibitor of lysosomal hydrolytic enzymes, markedly inhibited the degradation of internalized hCG (Ahmed and Niswender, 1981). Similar results were obtained in luteal cells from rats treated in vivo with chloroquine (Faircloth et al., 1983). The decrease in gonadotropin degradation induced by chloroquine was not due to general toxic effects, because cell viability during exposure to this inhibitor was maintained. Recently, the time course for accumulation of immunoactive hCG in subcellular compartments of luteinized ovaries obtained from hCG-treated rats indicated that as hCG associated with a combined plasma membrane/Golgi fraction declined, immunoactive hCG associated with lysosomal membranes increased, reaching a peak 10 to 14 hours post-injection (Gilligan et al., 1986). The membrane associated hCG retained its biological activity, while lysosomal hCG was not bioactive at any time
studied. These researchers also detected partially inactivated hCG in the cytosol that did not appear to be of lysosomal origin because its appearance in this compartment preceded the peak of lysosomal hCG and retained some bioactivity. Cytosolic hCG could represent hormone modified in prelysosomal compartments and/or at the cell surface with subsequent delivery to the cytosol. However, degradation of internalized gonadotropin by the lysosomal compartment of luteal cells remains the major route by which hormone is inactivated.

The fate of the receptor subsequent to internalization remains equivocal, due mainly to the lack of an adequate technique to specifically label it prior to or after binding of gonadotropin. Indirect methods, although admittedly less precise, appear to suggest that the receptor is recycled back to the plasma membrane of luteal cells. Treatment of ovine luteal cells with cyclohexamide, an inhibitor of protein synthesis, was without effect on the LH receptor number calculated by the summation of membrane-bound, internalized and degraded hCG (total-receptor-associated hCG) or by Scatchard analysis over a 48-hour period post-exposure to labeled hCG (Suter and Niswender, 1983). Receptors for LH were down-regulated over the 48 hours in control luteal cells not exposed to cyclohexamide. Furthermore, the amount of labeled hCG processed at the end of 48 hours was 2-fold greater than the amount of labeled hCG necessary to
saturate all available LH receptors at time zero. Suter and Niswender (1983) postulated that because cyclohexamide did not decrease LH receptor number, synthesis of new receptors was unnecessary for the continuance of binding, internalization and degradation of hCG. They provided two interpretations for these data: 1) luteal cells may possess a pool of preformed receptors that become available for binding over time, possibly the hidden receptors hypothesized to become unmasked during up-regulation; and 2) luteal cells may recycle LH receptors, possibly by exocytosis of secretory granules during progesterone secretion, as discussed previously. In support of the recycling hypothesis, specific, high-affinity LH receptors were identified in rough endoplasmic reticulum, heavy, medium and light Golgi, nuclear and lysosomal membranes from bovine CL (Rao et al., 1981; Rao et al., 1983) that were not attributable to contamination of the various fractions by subcellular organelles. The binding characteristics of all but the nuclear and lysosomal LH receptors were very similar to those of the plasma membrane. The lysosomal membranes exhibited a heterogeneous population of LH receptors and a slower $k_a$ than plasma membrane LH receptors. Therefore, if recycling of receptors does occur, processing of the receptors beyond the lysosomes should restore the binding properties back to those observed in plasma membranes. When hCG was bound to lysosomal preparations, eluted with acid and subsequently
exposed to fresh lysosomal membranes, it was able to rebind to receptors. The lack of hCG degradation in isolated fractionated lysosomal membrane indicated that the LH receptor was oriented to the external or cytosolic surface (Rao et al., 1981). The precise pathway whereby lysosomal associated receptors are recycled is unknown, but could presumably be inserted back into rough endoplasmic reticulum and/or Golgi membranes prior to exocytosis of the Golgi-derived vesicles. Alternatively, gonadotropin delivered to the lysosome during internalization could dissociate from the receptor at the lysosome, allowing the receptor to recycle back into other intracellular membranes. Until the hCG receptor itself can be specifically labeled without labeling of bound gonadotropin, the cellular fate of the receptor during the process of down-regulation will remain an enigma.

An alternative explanation for the loss of LH receptors during down-regulation has been proposed to involve receptor proteolysis. Labeling of rat ovarian membranes with tritiated borohydride, with subsequent binding of unlabeled hCG, revealed that two tritiated polypeptides of Mr = 69,000 and 38,000 were released into the media during a 2-hour period at 37 C (Kellokumpa and Rajaniemi, 1985). These researchers propose that the two polypeptides represent proteolytic fragments of the LH receptor because: 1) detergent solubilized receptor from rat ovarian membranes in the presence of N-ethylamide (NEM),
a protease inhibitor, migrated as a $M_r = 90,000$ polypeptide, while in the absence of NEM the two 69,000 and 38,000 polypeptides were observed; 2) thiol-blocking agents selectively prevented the appearance of the larger polypeptide; 3) metal-chelating agents decreased the appearance of the smaller component; and 4) these identical observations were observed when purified plasma membranes were used instead of solubilized receptor. Polypeptides of similar molecular weights have been identified during studies of LH receptor purification (Ji et al., 1981; Hwang and Menon, 1984; Bruch et al., 1986). Thus, the possibility exists for endogenous membrane proteolysis of the occupied LH receptor to partially account for down-regulation. Recently, Roche and Ryan (1986) identified multiple membrane proteases in luteinized rat ovaries of unknown physiological function. Additional studies utilizing whole luteal cells and evidence for dependence of the endogenous membrane proteases on LH/hCG will be required before proteolysis of occupied LH receptors can account for down-regulation of LH receptors.

Current experimental evidence suggests that the internalization of LH/hCG represents a degradation process, rather than a route whereby internalized hormone could be acting intracellularly to evoke a biological response. Progesterone synthesis in ovine luteal cells was unaffected by chloroquine, an agent shown to inhibit
degradation of hCG (Ahmed and Niswender, 1981). In addition, exposure of ovine luteal cells to concanavalin A resulted in increased internalization of hCG, but was without effect on progesterone synthesis (Ahmed and Niswender, 1981). Covalent cross-linking of rat ovarian membrane proteins by disuccinimidyl suberate, which presumably results in immobilization of membrane components to prevent internalization of LH-receptor complexes, also did not affect basal progesterone production (Hwang, 1983). Stimulation of luteal cell function by LH/hCG is thus manifested by interaction with the plasma membrane receptor and need not necessarily occur by a direct intracellular mechanism of action.

Activation of Adenylate Cyclase by LH-Receptor Complexes

The second major step involved in the sequence of events initiated by LH in the luteal cell is the transmission of the LH-receptor binding signal through the plasma membrane by the activation of adenylate cyclase. This enzyme catalyzes the conversion of ATP to cAMP which is necessary for steroidogenesis. The activity of adenylate cyclase has been monitored throughout the ovarian cycle in rat (Hunzicker-Dunn and Birnbaumer, 1976a), rabbit (Hunzicker-Dunn and Birnbaumer, 1976b), primate (Eyster et al., 1985) and bovine (Garverick et al., 1985) luteal tissue. In all of these species, basal and LH-stimulated adenylate cyclase activity was posi-
tively correlated with luteal progesterone production which also corresponded to the levels of LH receptors. Natural (Eyster et al., 1985; Garverick et al., 1985) and PGF₂α-induced lyteolysis (Agudo et al., 1984) was accompanied by decreases in basal and LH-stimulated adenylate cyclase activity. Exposure of ovine luteal tissue to LH and PGF₂α in vitro resulted in an increase and decrease, respectively, of adenylate cyclase activity (Fletcher and Niswender, 1982). Small ovine luteal cells were shown to exhibit an LH-stimulatable adenylate cyclase that correlated with in vitro progesterone synthesis, whereas large cells did not respond to LH with an increase in this enzyme (Hoyer et al., 1984).

An elegant and complete treatise regarding the structure and regulation of the adenylate cyclase system has been recently presented by Birnbaumer et al. (1985), the salient features of which will be presented below. References pertinent to the adenylate cyclase system of luteal cells will be included where appropriate.

At the center of the process by which the occupied hormone receptor is coupled to cyclase activity are two signal transducing proteins referred to as N (or G) proteins. One of these proteins, N₃, is responsible for mediating effects of hormones that stimulate cyclase activity, whereas the other, N₁, mediates the effects of inhibitory hormone receptors. The effects of LH on luteal cells have been shown to involve N₃ (Abramowitz and Birn-
Recently, the existence of an $N_i$ regulated by receptors that bind opioid analogues has been reported in rabbit luteal membranes (Abramowitz and Campbell, 1983). Both $N$ proteins bind guanine nucleotides and Mg and require these agents for their action. In addition to the $N$ proteins, the adenylate cyclase system contains a catalytic unit, called $C$, which forms cAMP plus MgPP$_i$ from the substrate MgATP.

The $C$ protein has been isolated and appears to have a $M_r = 150,000$. Little is known about the $C$ protein. Both $N$ proteins have been isolated, and while they are distinct in their functions, they possess structural similarities. Each is a trimer composed of $\alpha$, $\beta$ and $\gamma$ subunits. The $\alpha_S$ is heterogeneous with respect to its molecular weight, with a predominant form of $M_r = 42,000$ and a lesser form with $M_r = 51,000$. The $\alpha_S$, as well the the $\alpha_i$, bind GTP and its analogues. The $\alpha_i$ has a $M_r = 40,000$. The $\alpha_S$ can be ADP-ribosylated by cholera toxin, while the $\alpha_i$ is ADP-ribosylated by pertussis toxin. The $\beta$ and $\gamma$ subunits of $N_S$ and $N_i$ are identical and of $M_r = 35,000$ and 5000, respectively. It is presumed that the $\beta\gamma$ dimer binds Mg. The $N$ proteins possess GTPase activity.

Stimulation of $C$ by $N_S$ has been postulated to occur by interaction of $C$ with the "activated" $\alpha_S$-GTP complex of $N_S$ which requires GTP and Mg. The rate-limiting step in the conversion of basal $N_S$ to activated $N_S$ in luteal cells...
has been shown to be Mg-dependent binding of GTP to $\alpha_S$ (Abramowitz and Birnbaum, 1982). The rate of activation of $N_S$ and the total proportion of $N_S$ activated can be increased when Mg ion concentrations are increased. The apparent $k_m$ of $N_S$ for GTP is much less than the intracellular levels of GTP; therefore $N_S$ is always saturated with GTP. This, however, does not lead to stimulation of the cyclase system in the absence of hormone because $N_S$ is a GTPase and therefore degrades its own ligand, and the apparent $k_m$ for Mg is much greater than the intracellular Mg levels. It has been shown using purified $N_S$ that upon binding with GTP analogues, $\alpha_S$-GTP dissociates from the $N_S$ complex yielding $\beta\gamma$, and that the $\alpha_S$-GTP stimulates the $C$ protein. It is not yet known whether this dissociation of the activated $N_S$ occurs in mammalian plasma membranes. Thus, in the presence of GTP, $N_S$ binds GTP in a Mg-dependent manner. $N_S$ hydrolyzes this GTP by virtue of its intrinsic GTPase activity, and $N_S$ becomes deactivated with GDP bound to it which it can readily exchange for GTP to resume the activation cycle. Substitution of GTP by a nonhydrolyzable analogue such as GMP-P(NH)P maintains $N_S$ in an equilibrium between activated and unactivated form, thus abolishing the cyclical turnover of $N_S$ states.

Regulation of adenylate cyclase activity by stimulatory hormones has been demonstrated to involve the following sequence of events (Iyengar and Birnbaum, 1982). Hormone-receptor complexes increase the affinity
of \( N_s \) for Mg such that \( N_s \) now becomes saturated with Mg at physiologic, intracellular levels. This leads to an increase in the steady state levels of activated \( N_s \)-GTP (presumably \( \alpha_s \)-GTP). Thus, hormonal stimulation decreases the overall requirement of the cyclase system for Mg, such that the receptors can be considered "Mg switches."

In addition to this mechanism, stimulatory hormone action could also increase the absolute levels of activated \( N_s \) over that attained with saturating Mg. This appears to be achieved through the exchange of GDP for GTP by \( N_s \). Gonadotropins such as LH and hCG have been shown to stimulate this exchange reaction in Leydig cells (Dufau et al., 1980). This may be the more dominant mechanism whereby increases in steady-state levels of \( N_s \) are achieved by gonadotropin stimulation since the alteration in Mg affinity of \( N_s \) is not as great as observed with other stimulatory hormones in other tissues.

Activation of \( N_s \) by stimulatory hormones is also associated with an increased GTP hydrolysis. The hormone-receptor complex does not appear to alter the intrinsic properties of the \( N_s \) GTPase, but rather GTP hydrolysis is a reflection of increased cycling of the increased level of activated \( N_s \).

Reconstitution of purified \( N_s \) with purified hormone receptors in phospholipid vesicles showed for the first time that the only two proteins necessary for hormone-dependent stimulation of GTP-hydrolysis in \( N_s \) are the
receptor and $N_s$. It was also shown that the relative concentrations of both receptor and $N_s$ in the vesicles were important in regulating GTP hydrolysis.

Birnbaumer et al. (1985) provide evidence for the existence of two states of the stimulatory hormone receptors, one of high affinity and one of low affinity. The high-affinity state is achieved by agonist-receptor complexes, whereas the low-affinity state is observed when the receptor is occupied by an antagonist. For stimulatory hormones that bind to their receptors in a reversible fashion, both GTP and Mg are required for the receptor to attain the high affinity state. Activation of cyclase is achieved only by $N_s$ interaction with high-affinity receptors. Receptors for LH interact with LH in a rather irreversible manner, and as a consequence do not require GTP for the formation of the high-affinity state (Abramowitz and Birnbaumer, 1982). The most likely mechanism whereby Mg alters receptor affinity is upon Mg binding to $N_s$; the protein conformation of $N_s$ is altered such that $N_s$ interacts with the receptor to stabilize it in the high-affinity form.

While receptors behave apparently as two state systems, the N proteins have been shown to exist in three separate states. A description of these states will also serve to summarize the cycle by which stimulatory hormones activate adenylate cyclase. The first state of N, $N_s$-GDP, is capable of binding the hormone-receptor complex (HR),
forming HR-\(N_s\) with concomitant dissociation of GDP. The HR complex acts as a Mg switch, thereby increasing the affinity of \(N_s\) for Mg. Upon Mg saturation of \(N_s\), \(N_s\) adopts a new conformation (second state) whereby the HR is now transformed into a high-affinity state. Because GTP binding to \(N_s\) is Mg-dependent, the HR-\(N_s\)-GTP "activated" state is now attained. The HR-\(N_s\)-GTP is capable of activating C (third state), leading to the conversion of ATP to cAMP, as long as GTP remains unhydrolyzed. Upon expression of GTPase activity, the system would return to \(N_s\)-GDP, R of low affinity state, and C.

One last point of controversy should be addressed here. The model presented above by Birnbaumer et al. (1985) assumes the \(N_s\) and C proteins to be dissociated in the membrane, and only when HR-\(N_s\)-GTP is activated do the \(N_s\)-C associate to stimulate cyclase activity. However, Arad et al. (1984) provide evidence to indicate that the \(N_s\) and C are tightly associated at all times and that activation of cyclase involves only "collision coupling" between HR and \(N_s\)C. This issue awaits further clarification.

Many agents other than hormones can activate adenylate cyclase and are used in many experimental protocols to distinguish at which level of regulation, receptor-\(N_s\) interaction or C stimulation, various agonists and antagonists act on this system. The most commonly employed agents are NaF, forskolin and cholera toxin, in
addition to the nonhydrolyzable GTP analogs.

Fluoride ion, in the form of NaF, leads to increased cAMP production in all mammalian systems studied thus far, including luteal cells (Fletcher and Niswender, 1982). The actions of NaF lead to a persistent activation of $N_s$, similar to that seen with nonhydrolyzable GTP analogs, that is also dependent upon Mg. It is presumed that NaF causes the activated $N_s$ to dissociate to $a_s$-GTP which stimulates C.

Forskolin, a diterpene, also activates all adenylate cyclase systems studied to date, including ovine and primate luteal cyclase (Hoyer et al., 1984; Eyster et al., 1985), except that it is ineffective in sperm cells. Its precise mechanism of action is equivocal, but forskolin has been shown to stimulate solubilized C and therefore does not require an intact membrane. Therefore, it has been proposed that forskolin acts only on C. However, responsiveness to forskolin has also been shown to involve a protein, and is stimulated by, but not dependent on, the presence of $N_s$. Forskolin has been of value in identifying regulation of systems involving $N_i$ better than other agents (Abramowitz and Campbell, 1983), and has stimulated current interest in studying cyclase regulation.

Cholera toxin, secreted from the bacterium *Vibrio cholerae*, activates adenylate cyclase in an irreversible manner through the intracellular action of one of its
subunits, called A<sub>1</sub>. The A<sub>1</sub> peptide catalyzes the ADP ribosylation of an arginine residue in α<sub>S</sub> by the enzymatic transfer of ADP ribose from nicotinamide adenine dinucleotide (NAD; Moss and Vaughan, 1979). The addition of ADP ribose to α<sub>S</sub> blocks the GTPase activity of N<sub>S</sub>, resulting in persistent activation of adenylate cyclase.

Kohn (1978) has proposed that the glycoprotein hormone TSH might also activate thyroid adenylate cyclase using an ADP-ribosylating mechanism in view of the structural similarities between TSH and cholera toxin as well as their respective receptors. Because cholera toxin is presumed to be subverting a normal physiological mechanism, Kohn (1978) reasoned that it may be possible for TSH to use a different means of achieving ADP ribosylation of cyclase and/or proteins involved in the activation of cyclase by utilizing an NAD glycohydrolase activity which hydrolyzes NAD to nicotinamide and free ADP ribose in the absence of an acceptor protein or by the action of an ADP ribosyltransferase. Vitti et al. (1982) demonstrated that TSH increased the ADP ribosylation of bovine thyroid membrane proteins, and the major product ribosylated appeared to be the N<sub>S</sub> of adenylate cyclase. Thyroid stimulating hormone also ADP-ribosylated components that were released from the membranes into the supernatant of the reaction mixture, and one component was identified as the α subunit of TSH (Vitti et al., 1982). In addition, these researchers showed that the α subunit of TSH was
able to increase the intrinsic ADP ribosyltransferase activity of thyroid membranes, although it must be noted that it is difficult to obtain absolutely pure preparations of glycoprotein hormone subunits (Pierce and Parsons, 1981). Thus, TSH appears to ADP ribosylate the $N_s$ of adenylate cyclase through the action of an enzyme intrinsic to the thyroid plasma membrane, as well as "autoribosylating" its own $\alpha$ subunit. The production of cAMP was associated with increased ADP ribosyltransferase activity in the thyroid membranes. Rat thyroid membranes were shown to possess both ADP ribosyltransferase and NAD glycohydrolase activities that can be stimulated by TSH (DeWolf et al., 1981).

Cholera toxin also induced the ADP ribosylation of $\alpha_s$ in rabbit luteal plasma membranes, but in contrast to TSH in thyroid membranes, oLH did not appear to ADP ribosylate the $\alpha_s$ nor any other proteins (Abramowitz and Campbell, 1985). Similar results were observed in rat luteal membranes wherein cholera toxin stimulated ADP-ribose incorporation into $\alpha_s$, but hCG did not (McIlroy and Bergert, 1984; Roche and Ryan, 1986). The possibility that a hormone-responsive NAD glycohydrolase activity exists in luteal plasma membranes has not been investigated. Thus, a universal role for the ribosylation of membrane proteins by glycoprotein hormones, as well as a corresponding activation of adenylate cyclase, remains equivocal.
The fact that LH binds to its receptor tightly and in an irreversible manner leads one to question how deactivation of the adenylate cyclase system can occur. With other stimulatory hormones, the GTPase activity of Ns converts HR-Ns-GTP to HR plus Ns-GDP because of the GTP requirement for the high affinity state of HR. Recall that the LH receptor has no GTP requirement. The mechanism believed to turn off the system stimulated by LH has been termed desensitization. Continued occupancy of the LH receptor by LH is accompanied by a major decrease in cAMP production which occurs prior to the down-regulation of LH-receptor complexes by internalization (Hunzicker-Dunn and Birnbaumer, 1976c; Lamprecht et al., 1977). When adenylate cyclase loses its responsiveness to the hormone that induced the desensitization, the desensitization is called homologous. When responsiveness is lost to other stimulating hormones as well, heterologous desensitization occurs (Hunzicker-Dunn et al., 1979a; Kirchick and Birnbaumer, 1981). Desensitization is usually studied in luteal tissue exposed to high levels of LH in vivo, as would occur during the preovulatory surge, or high doses of LH or hCG in vitro. Under these conditions, saturating levels of gonadotropin are exposed to all receptors which eventually results in desensitization of the cyclase system.

Homologous desensitization appears to be caused by a functional uncoupling of the HR from activated Ns
resulting in a return of $N_s$ to its inactivated form followed by a fall in $C$ activity. This uncoupling is not due to a modification of $N_s$, because cholera toxin, NaF and GMP-P(NH)P were able to stimulate adenylate cyclase in desensitized luteal and follicular cells (Sen et al., 1979; Ezra and Salomon, 1980; Hunzicker-Dunn, 1981a). Homologous desensitization appears to require Mg and ATP (Bockaert et al., 1976). Hunzicker-Dunn et al. (1979b) proposed that homologous desensitization was a result of a $cAMP$-independent phosphorylation of the occupied hormone receptor based upon its Mg and ATP requirement and the ability of a phosphoprotein phosphatase to reverse the inhibition of adenylate cyclase activity. It was suggested that homologous desensitization of thyroid adenylate cyclase by chronic TSH exposure was the result of ADP ribosylation of all available acceptor proteins (Vitti et al., 1982).

Heterologous desensitization in rat luteal cells in response to catecholamine stimulation was assessed by reconstituting luteal $N_s$ with the cyc$^-$ variant of the S49 mouse lymphoma cell line which contains functional $\beta$-adrenergic receptors and $C$, but lacks $N_s$ (Kirchick et al., 1983). These researchers noted a decreased ability of the $N_s$ to be activated, but the nature of the $N_s$ modification and the mechanism by which it occurs are unknown.

Because of the relatively low tonic levels of circu-
lating LH during the luteal phase of the estrous cycle in domestic animals, it is possible that not all of the LH receptors are occupied (Diekman et al., 1978a; Ziecik et al., 1980; Garverick et al., 1985) to the saturating extent observed during ovulation. Although basal levels of adenylate cyclase activity remain rather high during luteal progesterone production (Marsh, 1970; Agudo et al., 1984; Hoyer et al., 1984), homologous desensitization may not be occurring. This phenomenon has not been studied extensively in ovarian tissues of domestic animals, yet early reports noted a refractoriness to LH in ovine CL in vivo (Baird and Collett, 1973) and bovine CL in vitro (Hansel, 1971). Recently, Bourdage et al. (1984) demonstrated that the continuous exposure of small ovine luteal cells to oLH in vitro resulted in decreased progesterone synthesis.

Homologous desensitization of adenylate cyclase activity occurs in cells in response to high levels of gonadotropins and involves some kind of alteration of N_s. The mechanism by which N_s is rendered incapable of stimulating C remains speculative. However, the recent observations that LH containing a deglycosylated α subunit can bind to the LH receptor without eliciting stimulation of cyclase (Sairam, 1983), and that deglycosylated LH can prevent LH-induced desensitization in luteal cells (Zor et al., 1984) may provide the basis for interesting future studies concerning the desensitization process.
cAMP-Dependent Protein Kinase Activation

Intracellular cAMP produced as a result of LH-stimulated adenylate cyclase activity has two fates; it can be degraded by a phosphodiesterase to 5'-AMP (Marsh, 1976), or it can bind to cAMP-dependent protein kinase (Kuo and Greengard, 1969). The central theme of the second messenger role of cAMP assumes that all of the intracellular effects of cAMP are mediated by cAMP-dependent protein kinases. Activation of these protein kinases catalyzes the phosphorylation of key regulatory proteins involved in the cellular responses elicited by the hormone.

The properties and regulatory mechanisms involved in the activation of cAMP-dependent protein kinases have been reviewed by Flockhart and Corbin (1982). Most of this protein kinase activity is located in the soluble cell fraction. The native form of protein kinase exists as a tetramer composed of two catalytic subunits and two regulatory subunits. The regulatory subunits are linked together by disulfide bonds, and each contain two cAMP binding sites. In the absence of cAMP, the holoenzyme is inactive. Activation of protein kinase occurs upon the binding of cAMP to the regulatory subunits, which promotes the dissociation of the catalytic subunits from the regulatory subunits. The free catalytic subunits then catalyze the phosphorylation of serine or threonine residues of various protein substrate(s), thus modifying the activity
of those responsible for causing cellular response.

It is unclear whether both cAMP binding sites on each regulatory subunit need to be occupied in order to release the catalytic subunits, although cAMP does interact with both sites during activation of protein kinase. The catalytic subunits also contain one binding site for ATP, and when occupied in vitro, higher concentrations of cAMP are required to stimulate dissociation of the regulatory subunits. The physiological significance of ATP binding in vivo has not been assessed. The regulatory subunits can also be phosphorylated by a cAMP-dependent protein kinase in vitro, which results in impaired association of this subunit with the catalytic subunit. Again, this observation has not been made in vivo. The association of the holoenzyme with cAMP is also a reversible reaction that exists in equilibrium inside the cell. Subunit dissociation and reassociation are not only dependent upon the intracellular cAMP concentration, but one or more heat-stable proteins have been shown to interact with the catalytic subunit rendering it inactive. It is also conceivable that the interactions of the holoenzyme with ATP and/or their different phosphorylation states may contribute to the reaction equilibrium.

Two types of cAMP-dependent protein kinase, Type I and Type II, have been identified and are distinct with respect to their regulatory subunits. Type I regulatory subunit (R') has a $M_r = 47,000$, whereas that of Type II
regulatory subunit (R") is 54,000, and R' and R" reveal major differences in their primary structure. The catalytic subunits of the two types appear to be identical. The regulatory and catalytic subunits of Type I also appear to dissociate more readily than those of Type II, and also to reassociate more slowly. Thus, less cAMP is necessary to dissociate the Type I holoenzyme than Type II. Nearly all tissues contain both Type I and Type II protein kinases, but the presence of one usually predominates over the other. There are also tissue and species variations in the distribution of these enzymes.

The type of cAMP-dependent protein kinase present in ovarian cells is dependent upon the reproductive state of the female. Mature preovulatory follicles from rabbits (Hunzicker-Dunn and Jungmann, 1978) and sows (Hunzicker-Dunn et al., 1979) contain predominantly the Type II protein kinase, although minute quantities of Type I are also present. In rat granulosa cells, R" predominates over R', but thecal cells express a similar abundance of both R" and R' (Richards and Rolfes, 1980). Upon luteinization subsequent to the preovulatory surge of LH, a dramatic increase in the levels of R' and Type I kinase are observed in rabbit (Hunzicker-Dunn and Jungmann, 1978; Hunzicker-Dunn, 1983) and porcine CL (Dimino and Bieszczad, 1982). The appearance of R' and Type I were also shown to be specifically induced by LH and/or hCG in CL from these species. The increase in R' was not accom-
panied by a decrease in the levels of R", so that CL contain significant levels of both protein kinases. Bovine CL (Menon, 1973) also exhibit two cAMP-stimulated protein kinase activities that presumably are the Type I and II enzymes. Recently, Hoyer and Niswender (1985) have demonstrated the presence of a Type I protein kinase in small ovine luteal cells. During luteolysis in rabbits (Hunzicker-Dunn and Jungmann, 1978) the level of Type I protein kinase almost disappears. The distribution of protein kinase in luteal cells obtained from pregnant animals has not yet been reported. The above results suggest that the Type I protein kinase is required for the expression of progesterone production in functional CL.

Generalized increases in intracellular cAMP levels induced by hormones and subsequent activation of protein kinase is expected to result in the phosphorylation of intracellular substrates for the kinase. Yet, some degree of specificity in cellular responses upon stimulation by different hormones that use the cAMP pathway must be maintained. It is possible that only a few substrates for cAMP-dependent protein kinases exist, but in vitro phosphorylation experiments usually reveal a multitude of phosphorylated proteins. However, the sole observation that a protein can be phosphorylated by cAMP in vivo is insufficient to conclude that the protein has a physiologically relevant function (Beavo and Mumby, 1982). Substrate specificity could be achieved through selective
activation of one form of cAMP-dependent kinase. The results of Hunzicker-Dunn (1981b) strongly suggest that only the Type I protein kinase is exposed to increased cAMP levels induced by hCG in intact rabbit luteal cells, and that Type II kinase activation regulates protein phosphorylation in follicles.Selective activation of protein kinase cannot be the sole explanation for substrate specificity because luteal cells still express Type II enzyme even though it is not regulated by hCG. Similarly, rat ovarian granulosa cells, known to respond to both FSH and LH, contain both R' and R" (Richards and Rolfes, 1980). Hayes and Brunton (1982) have hypothesized that substrate specificity may result as the consequence of cAMP production within specific cellular compartments, thereby activating protein kinase that would phosphorylate substrates only within that compartment. It is conceivable that such compartmentalization could involve hormone-induced changes in cellular cytoskeletal organization (Zor, 1983). The compartmentalization hypothesis is compatible with selective activation of protein kinase if the different types of kinase are sequestered in distinct locations within the cell.

The proteins phosphorylated in small bovine luteal cells in response to LH have been demonstrated to include species with Mr 64,000, 84,000, 93,000, 99,000 and 110,000 (Darbon et al., 1981). Within five minutes of LH treatment, both phosphorylation of these proteins and proges-
terone production were increased. Steroidogenic enzymes of luteal tissue were also regulated by protein kinase activation. Caron et al. (1975) demonstrated that cAMP-dependent protein kinase phosphorylation of bovine luteal proteins could enhance cholesterol side-chain cleavage activity in a cell-free system. Cholesterol esterase activity from ovine CL was shown to be increased in vitro upon exposure to LH and dibutyryl cAMP, indirectly suggesting regulation by cAMP-dependent protein kinase (Caffrey et al., 1979a). Cholesterol esterase in bovine adrenal cortex was indeed directly phosphorylated by cAMP-dependent protein kinase resulting in increased hydrolase activity and steroidogenesis (Beckett and Boyd, 1977).

Richards and Kirchick (1984) showed that LH, rather than increase the phosphorylation of substrate proteins, induced a rapid decrease in the amounts of specific proteins phosphorylated by FSH. Phosphorylation of actin, R" and four other proteins were observed in rat ovarian follicular cytosol exposed to dibutyryl cAMP. Phosphorylation of these proteins was markedly reduced by exposure of follicular cytosol to hCG, and was also low in luteal cytosol. Reduction in phosphorylation was not attributable to increases in cytosolic phosphoprotein phosphatase and ATPase activities. Thus, LH appeared to regulate the concentrations of protein kinase substrates.

In conclusion, LH-induced increases in intracellular cAMP concentrations lead to the phosphorylation of
specific proteins via the action of protein kinase. Some of these proteins may be directly involved in luteal steroidogenesis, whereas the identity and functions of others remain to be demonstrated. The precise mechanism(s) that determine substrate specificity of cAMP-dependent protein kinases in the ovary are not clearly understood, but could involve differential cellular compartmentalization of the Type I and II kinases and their respective substrates, as well as hormone-specific regulation of these protein kinases during different reproductive states.

Luteal Steroidogenesis

Progesterone biosynthesis in luteal tissue is mediated by LH-induced increases in cAMP production. The portions of the steroidogenic pathway that are regulated by LH in luteal cells include increasing the uptake of cholesterol from the plasma, the activity of steroidogenic enzymes, as well as the association of cholesterol with the mitochondrial side-chain cleavage enzyme. These events will be summarized below.

Progesterone is derived from cholesterol, which can be obtained by steroidogenic tissues via uptake from plasma lipoproteins, de novo synthesis from acetyl CoA, or the hydrolysis of cholesteryl esters stored in lipid droplets within the cytoplasm (Marsh, 1976). The uptake of lipoprotein complexed with cholesterol esters appears to be the major source of cholesterol utilized by luteal
cells (for review, see Gwynne and Strauss, 1982). The two major circulating lipoprotein particles are the low-density and high-density lipoproteins (LDL and HDL, respectively) which consist of a core of neutral lipids, triglycerides and cholesteryl esters, surrounded by a coat of phospholipids and proteins. The composition of LDL differs from HDL in that it contains a higher percentage of cholesterol, and contains an apoprotein designated as apoprotein B. The HDL's consist of three major classes that also differ among themselves with respect to their apoprotein composition of apo A-I, apo A-II, and apo E. The apoproteins of LDL and HDL determine the specificity of binding of the lipoproteins to plasma membrane receptors. Distinct receptors for LDL and HDL have been demonstrated in the rat CL, and upon stimulation by hCG in vivo, an increase in the number of both types of receptor is observed (Hwang and Menon, 1983). Subsequent to the binding of lipoprotein, the lipoprotein-receptor complexes migrate to coated pits in the membrane, are internalized by endocytosis and degraded by lysosomal enzymes to yield free cholesterol (Goldstein et al., 1980; Rajendran et al., 1983). Treatment of rats in vivo with 4-aminopyrazolo [3,4-d]pyrimidine (4-APP), an adenine analog which causes reduced plasma cholesterol, decreased cholesteryl ester content and increased de novo cholesterol synthesis in the ovaries (Anderson and Dietschy, 1978). Infusion of HDL into these rats restored ovarian
levels of cholesterol. These studies indicate that lipoprotein uptake may be the major source of cholesterol for steroidogenesis. In addition, the exposure of luteal cells in vitro to HDL and LDL greatly augmented basal and hCG-stimulated progesterone production (Ahzar and Menon, 1981; Schuler et al., 1982a). Although data regarding LH regulation of HDL and LDL receptors in ovine CL are not available, the experiments of Hoyer and Niswender (1985) indicate that the availability of cholesterol influenced by lipoprotein uptake could be an LH-induced mechanism active in small cell steroidogenesis. Progesterone production by large cells was not enhanced by exogenous cholesterol substrates.

De novo cholesterol synthesis occurs by the enzymatic conversion of acetate, in the form of acetyl-CoA. Activity of the rate-limiting enzyme, hydroxymethylglutaryl CoA reductase, increases when intracellular cholesterol stores are low, as was demonstrated in rat CL (Schuler et al., 1979). Stimulation of progesterone production by LH was not due to a stimulation of reductase activity (Schuler et al., 1981b).

The amount of cholesterol stored as cholesteryl esters in lipid droplets within luteal cells is regulated by two enzymes, cholesterol esterase and cholesterol synthetase. The activity of cholesterol esterase has been shown to be increased in the presence of LH in ovine (Caffrey et al., 1979a) and bovine (Marsh, 1976) CL. The
cholesterol esterase of bovine CL displays a requirement for cAMP and possibly protein kinase, although this was demonstrated indirectly (Bisgaier et al., 1979). However, bovine adrenal cortex cholesterol esterase was directly phosphorylated by a cAMP-dependent protein kinase (Beckett and Boyd, 1977). Free cholesterol ester has been detected in ovarian tissue as a result of a LH-stimulated inhibition of cholesterol synthetase. Marsh (1976) suggested that the effects of LH on cholesterol ester hydrolase and synthetase are probably secondary responses which maintain intracellular levels of free cholesterol during accelerated progesterone synthesis.

Transport of free cholesterol to the mitochondria is necessary prior to its ultimate conversion to progesterone. Mori and Marsh (1982) demonstrated that LH increased the accumulation of cholesterol in mitochondria of rat CL. This appeared to be a primary effect of LH, and not due to stimulation of steroidogenesis at some later point in the pathway because LH-induced cholesterol accumulation still occurred in the presence of aminoglutethimide, a drug that inhibits the conversion of cholesterol to pregnenolone. Cholesterol accumulation was evident in the presence of cyclohexamide, therefore de novo synthesis of new proteins was not involved in transport to the mitochondria. Cholesterol transport does require the action of cytoskeletal components, particularly microfilaments, because cytochalasin B blocks this
LH-induced effect in ovine CL (Silavin, et al., 1980). In addition to gonadotropin-induced cholesterol accumulation, LH facilitates the conversion of a portion of this cholesterol into a discrete pool that is used for steroidogenesis in rat CL (Toaff et al., 1979; Mori and Marsh, 1982). This pool of cholesterol is redistributed from the outer to the inner mitochondrial membrane where the initial step in progesterone synthesis occurs, and is facilitated by an LH-induced labile protein (Toaff et al., 1979). This appears to be similar to the sterol carrier protein responsible for the redistribution of mitochondrial cholesterol in adrenal glands (Simpson et al., 1978; Privale et al., 1983).

The rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by the side chain cleavage enzyme. Side chain cleavage is located in the inner mitochondrial membrane in adrenal (Kimura, 1981) and bovine luteal tissue (Kashiwagi et al., 1980). Side chain cleavage activity binds cholesterol and catalyzes sequential hydroxylations at carbons 22 and 20, followed by cleavage of the C20-C22 bond to yield pregnenolone and isocaproaldehyde. This reaction requires 3 NADPH and 3 O2 that are transported via ferridoxin reductase, ferridoxin and cytochrome P-450. The binding of the hydroxylated intermediates to the enzyme is much higher than that of cholesterol, ensuring completion of the reaction (Lambeth et al., 1982). Using an in vitro reconstitution system,
Caron et al. (1975) demonstrated a cAMP-dependent phosphorylation of bovine luteal side chain cleavage that resulted in the activation of this enzyme, suggesting induction of steroidogenesis by LH. Only recently it has been shown that the activity of side chain cleavage is enhanced by LH in rat luteal mitochondria (Mori and Marsh, 1982).

The final step in the steroidogenic pathway of luteal cells is the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD; Marsh, 1976; Caffrey et al., 1979b). Presumably pregnenolone is transported from the mitochondria to the smooth endoplasmic reticulum where 3β-HSD is localized in ovine luteal cells (Caffrey et al., 1979b). The conversion of pregnenolone to progesterone does not appear to be rate limiting for steroidogenesis, and therefore is probably not regulated by LH.

In summary, luteal progesterone synthesis is regulated by LH at a number of loci in the steroidogenic pathway including increasing HDL and LDL receptors resulting in increased cholesterol uptake, increased transport of cholesterol to and within mitochondria, increased activation of side chain cleavage, and increased cholesterol esterase activity. Among these, the stimulation of side chain cleavage and cholesterol esterase activities appear to involve phosphorylation by a cAMP-dependent protein kinase. It is not known whether the
other LH-regulated proteins involve a dependency upon cAMP for their actions of cAMP on cytoskeletal function (Hall, 1982) and initiation of protein synthesis (Ahzar and Menon, 1975; Marsh, 1976).

The diminished activity of the LH-regulated loci in progesterone production during desensitization in luteal cells exposed to high levels of hCG in vitro was suggested by Rajendran et al. (1985). These researchers demonstrated that lipoprotein uptake by luteal cells was not impaired during desensitization. This suggests that the conversion of cholesterol to progesterone by the mechanisms summarized above are controlled by LH-induced activation of adenylate cyclase and protein kinase activity.

Role of the Plasma Membrane in Luteal Cell Function

Structural and Functional Aspects of Plasma Membranes and Plasma Membrane Fluidity

The ovine luteal cell receives a variety of extracellular hormonal signals during the distinct reproductive stages associated with the decline of progesterone production in late diestrus or with the preservation of steroidogenesis by the presence of the conceptus during early pregnancy. The transmission of these signals initiated by the specific hormone-receptor interaction occurs across the luteal cell plasma membrane and may involve numerous
events localized within this organelle. The elucidation of plasma membrane structure and dynamics offers an exciting, and as yet relatively unexplored, area of investigation that will contribute significantly to our understanding of the regulation of ovine luteal cell function by LH, prostaglandins and embryonic luteotropins.

Major advances in the acquisition of knowledge concerning plasma membrane dynamics have been based upon the now universally accepted fluid mosaic model of membrane structure introduced over a decade ago by Singer and Nicolson (1972). The fluid mosaic model encompasses a number of basic principles concerning the gross organization and structure of the proteins and lipids to which biological membranes are assumed to conform. These principles include: 1) the bulk membrane lipids, represented principally by the phospholipids and their corresponding fatty acids, are arranged in a planar, bilayer configuration that is predominantly in a fluid state under physiological conditions; 2) the lipid bilayer exists in a discontinuous configuration whereby interruptions are due to the presence of peripheral and integral membrane proteins and glycoproteins, the majority of which are represented as oligomeric complexes rather than individual peptide components; 3) the organization of the lipids, glycolipids, proteins and glycoproteins is distributed asymmetrically within the membrane such that they are exposed either at the inner or outer membrane surface;
and 4) the proteins and glycoproteins are heterogeneous with respect to their size, structure, location and mobility (Singer and Nicolson, 1972; Nicolson, 1979).

The existence of plasma membranes in a predominantly fluid state under physiologic conditions carries important structural and functional implications. Cell types responsive to stimulatory hormones act through receptors that are integral or peripheral plasma membrane proteins leading to the stimulation of adenylate cyclase activity. The plasma membrane of the target cell not only provides the appropriate structural environment necessary to confer the proper receptor conformation and stability required for hormone binding, but its inherent fluidity may also determine the coupling of the hormone-receptor complexes with adenylate cyclase, endocytosis of hormone-receptor complexes, the exocytosis of secretory products and the insertion of newly synthesized or recycled hormone receptors.

A logical prerequisite to understanding plasma membrane function is having knowledge concerning its composition. Unfortunately, there are no reports in the literature describing the composition of CL plasma membranes from domestic animals. A paucity of information regarding the lipid composition of mammalian ovaries exists in comparison to the abundant characterization of testicular lipids (Coniglio, 1977), and even less is available with respect to the CL. Summaries of the most
comprehensive phospholipid and fatty acid analyses of mammalian ovarian tissues are presented in Tables 3 and 4, respectively. Because the majority of these analyses are derived from total ovarian or luteal extracts, it is impossible to draw specific conclusions regarding the lipid composition of the plasma membrane. In view of the fact that the principal components of plasma membranes are the phospholipids and their respective fatty acids (Singer and Nicolson, 1972), the composition of these lipids in ovarian tissues may be useful in providing very general observations regarding ovarian plasma membranes. However, luteal tissue also contains an abundant network of smooth endoplasmic reticulum due to its steroidogenic function, therefore phospholipid analyses reflect more closely the combined compositions of the surface and intracellular membranes. The following generalizations can be made. First, the phospholipid composition of mammalian ovaries, including that of bovine CL, contains phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as the major phospholipid species, with phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SPH) and cardiolipin. These are also the major phospholipids present in most mammalian cell plasma membranes (Ansell et al., 1973). A comparison of the fatty acid composition from total lipids, neutral lipids and phospholipids of ovaries is presented in Table 4. The principal fatty acids in these tissues are palmitic (16:0), stearic (18:0), oleic (18:1),
Table 3. Summary of the Phospholipid Composition of Mammalian Ovaries

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Rabbit&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rat&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Bovine&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>17.5</td>
<td>17.9</td>
<td>53.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>14.1</td>
<td>8.9</td>
<td>13.6</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>2.1</td>
<td>0.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>6.8</td>
<td>1.3</td>
<td>4.6</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>nd</td>
<td>nd</td>
<td>0.8</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>nd</td>
<td>nd</td>
<td>3.9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>2.3</td>
<td>1.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Lysophosphatidyl choline</td>
<td>1.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Choline plasmologen</td>
<td>nd</td>
<td>nd</td>
<td>1.7</td>
</tr>
<tr>
<td>Ethanolamine plasmologen</td>
<td>nd</td>
<td>nd</td>
<td>8.5</td>
</tr>
</tbody>
</table>

nd = not determined

<sup>a</sup> = mg phospholipid/g ovarian dry weight; Morin (1968)
<sup>b</sup> = μmol P/g ovarian wet weight; Strauss and Flickinger (1977)
<sup>c</sup> = % of total phospholipid P in CL; Scott et al. (1968)
Table 4. Summary of the fatty acid composition of mammalian ovaries

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Bovine ovarya&lt;sup&gt;a&lt;/sup&gt; Total</th>
<th>Neutral</th>
<th>PL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Bovine CL&lt;sup&gt;b&lt;/sup&gt; Total</th>
<th>Neutral</th>
<th>PL</th>
<th>Porcine ovarya&lt;sup&gt;a&lt;/sup&gt; Total</th>
<th>Neutral</th>
<th>PL&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Rat ovary Polare&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PL&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Ovine CL&lt;sup&gt;c&lt;/sup&gt; PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.5</td>
<td>1.2</td>
<td>0.8</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>4.2</td>
<td>0.5</td>
<td>nd&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>14.6</td>
<td>14.8</td>
<td>11.9</td>
<td>16.8</td>
<td>11.4</td>
<td>17.8</td>
<td>28.6</td>
<td>20.0</td>
<td>23.8</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>16:1</td>
<td>2.5</td>
<td>1.9</td>
<td>5.0</td>
<td>2.8</td>
<td>1.1</td>
<td>1.3</td>
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<tr>
<td>18:0</td>
<td>14.1</td>
<td>15.3</td>
<td>9.7</td>
<td>12.0</td>
<td>13.0</td>
<td>16.0</td>
<td>19.3</td>
<td>18.2</td>
<td>14.3</td>
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<td></td>
</tr>
<tr>
<td>18:1</td>
<td>15.3</td>
<td>10.3</td>
<td>27.0</td>
<td>18.6</td>
<td>14.2</td>
<td>10.9</td>
<td>9.8</td>
<td>11.2</td>
<td>21.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>16.5</td>
<td>12.1</td>
<td>11.3</td>
<td>14.6</td>
<td>13.4</td>
<td>10.2</td>
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<tr>
<td>18:3</td>
<td>1.3</td>
<td>1.8</td>
<td>1.9</td>
<td>1.3</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>2.5</td>
<td>9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:2</td>
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<td>2.4</td>
<td>3.0</td>
<td>1.6</td>
<td>1.4</td>
<td>0.8</td>
<td>0.2</td>
<td>0.9</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:3</td>
<td>3.7</td>
<td>3.1</td>
<td>1.7</td>
<td>2.7</td>
<td>1.9</td>
<td>1.8</td>
<td>1.3</td>
<td>nd</td>
<td>nd</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20:4</td>
<td>13.4</td>
<td>13.0</td>
<td>7.2</td>
<td>13.8</td>
<td>26.2</td>
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<td>15.2</td>
<td>14.2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>22:4</td>
<td>6.4</td>
<td>5.3</td>
<td>4.4</td>
<td>3.5</td>
<td>12.3</td>
<td>6.4</td>
<td>5.4</td>
<td>16.1</td>
<td>2.7</td>
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<td>8.8</td>
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<td>2.3</td>
<td>3.2</td>
<td>0.5</td>
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<tr>
<td>22:6</td>
<td>trace</td>
<td>0.0</td>
<td>trace</td>
<td>trace</td>
<td>0.8</td>
<td>3.6</td>
<td>1.2</td>
<td>2.1</td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> % of total lipid or phospholipid; Holman and Hofstetter (1965)

<sup>b</sup> % (w/w); day 9-13 of estrous cycle; Scott et al. (1968)

<sup>c</sup> % (w/w); day 12 of estrous cycle; Waterman (1980b)

<sup>d</sup> PL = phospholipid

<sup>e</sup> % (w/w); 3 days post-hCG treatment; Strauss and FLickinger (1977)

<sup>f</sup> % (w/w); 14 days post-hCG treatment; CL microsomal membranes; Carlson et al. (1981)

<sup>g</sup> nd = not determined
linoleic (18:2) and arachidonic (20:4) acid. It is interesting to note that the phospholipid fatty acid compositions in ovarian tissues, including CL, are very similar and that the phospholipid fatty acid composition of rat CL microsomal membranes (Carlson et al., 1981) reflects that observed in total ovarian phospholipids. In addition, microsomal membranes from bovine CL revealed that PC was the major phospholipid, representing 68% of the total phospholipid, and that 69% of the total fatty acids consisted of 16, 18 and 20 carbons (Goodsaid-Zalduondo et al., 1982).

The role of phospholipids on hCG-binding to bovine luteal membranes has been examined (Ahzar and Menon, 1976; Ahzar et al., 1976). Exposure of purified membranes to phospholipase A, which cleaves a fatty acid from the phospholipid yielding lysophospholipid, inhibited hCG-binding by decreasing the number of available receptors. Treatment with phospholipase C, which cleaves the polar head group from the phospholipid yielding diacylglycerol, also decreased LH receptors. Neither phospholipase affected the Kd, nor did they affect the binding of preformed hCG-receptor complexes (Ahzar and Menon, 1976). These results suggested that the binding inhibition caused by phospholipase A was due to a direct effect on the phospholipids present in the immediate vicinity of the receptor or to an inhibitory effect of the resultant lysophospholipids. The inhibition caused by phospholipase C suggested that the
Polar head groups were necessary for hCG-binding. Pretreatment of bovine luteal membranes with phospholipase A or C followed by the addition of liposome suspensions containing various phospholipids did not restore gonadotropin binding activity (Ahzar et al., 1976). The polar head groups of PC, PE and PS, in addition to diacylglyceride, had no effect on hCG receptor activity. In contrast, lyso PC, lyso PE and lyso PS inhibited the ability of both membrane-associated and solubilized LH receptors to bind hCG. Washing the receptor preparations with defatted bovine serum albumin to remove the lysophospholipids restored the receptor binding activity. These results did not indicate a requirement of the LH receptor for the phospholipid polar moieties and suggested further that the presence of lysophospholipids may mask portions of the receptor necessary for binding or directly interfere with the binding of hCG to the receptor. Reconstitution of the purified, solubilized receptor with liposome suspensions made from either PE or PS restored the ability of the receptor to bind hCG, indicating a possible role for these phospholipids in maintaining the LH receptor in a proper physical conformation conducive to hormone binding. Similar reports indicated the importance of phospholipids in the interaction between FSH with testicular membrane receptors (Abou-Issa et al., 1976; O’Neill and Reichert, 1984).

In contrast, TSH binding to thyroid hormone receptors
was inhibited by PI, PE and PS (Aloj et al., 1979). The nature of this inhibition seemed to be due to an interaction between the phospholipid and TSH. It was shown that PI formed an adduct with TSH that was incapable of binding to the receptor. Liposomes containing PC and cholesterol could substitute for the intact thyroid plasma membrane with respect to the binding of TSH to the glycoprotein component of the TSH receptor. The addition of PI to this liposome abolished receptor binding. Thus, in the thyroid membrane, phospholipids (particularly PI) act as negative modulators of TSH receptor expression by affecting some unknown property of the TSH molecule.

Specific membrane phospholipids may also play a key role in the hormone-induced activation of adenylate cyclase (Levey and Lehotay, 1976). Solubilized adenylate cyclase from cat myocardium loses its ability to be activated by hormones, but not by NaF. Glucagon-stimulated adenylate cyclase activity was restored when PS was added to the solubilized enzyme. Similarly, PE was effective in restoring catecholamine responsiveness to soluble cardiac adenylate cyclase. The stimulatory effects of these phospholipids appeared to be manifested at the level of $N_s$ because they had no effect on hormone binding. Similarly, the glucagon-responsiveness of liver adenylate cyclase that had been exposed to phospholipase A was restored with suspensions of PS. Phospholipase A-treated thyroid membranes responded to TSH with increased adenylate
cyclase activity in the presence of PC suspensions. Shier et al. (1976) examined the effect of various preparations of lyso PC and discovered that it was a potent inhibitor of NaF-stimulated adenylate cyclase activity in 3T3 mouse fibroblasts. The inhibition was apparently not attributable to any portion of the lyso PC structure, but appears to result from the surfactant properties of this lipid.

In addition to the structural role the plasma membrane possesses necessary for hormone binding and activation of adenylate cyclase, its fluid physical properties may play an important part in the regulation of cellular function. Alterations in membrane fluidity leading to changes in cell function can be influenced in part by the lipid composition of the membrane. Comparing the fluid properties from a wide variety of cell membranes exposed to various conditions has led to some generalizations with regard to the compositional changes associated with membrane fluidity (Thompson, 1980; Shinitzky, 1984). In most cases, the lipids that make up the bulk matrix of the plasma membrane in mammalian cells are composed of predominantly fluid components due to a considerable degree of phospholipid acyl chain motion under most physiological systems. Bulk fluidity can be influenced by unesterified cholesterol which acts to induce "rigidity" by reducing lateral molecular spacing and the flexibility of phospholipid acyl chains (Shinitzky, 1984). Cholesterol appears to have a dual effect on lipid systems in that at a
temperature where the lipids would be in a gel phase, the presence of cholesterol causes the lipids to become fluid, whereas at a temperature when the lipid is in a fluid condition, cholesterol inhibits some of the acyl chain motion. Unesterified cholesterol appears to be the principal effector of lipid fluidity in biological membranes (Shinitzky, 1984). Bulk membrane fluidity can also be increased by (in order of significance): increased degree of unsaturation of the phospholipid acyl chains, decreased length of the acyl chains, decreased mole ratio of SPH:PC, decreased mole ratio of PE:PC and increased ratio of protein:lipid (Shinitzky, 1984).

The bulk fluidity characteristics of plasma membranes exist in two main phases called the liquid-crystalline phase, which represents the fluid state of membranes, and the gel phase, which represents a more rigid or solid state of the membrane (Thompson, 1980). Each lipid in a membrane has its own characteristic phase transition temperature, or melting point, that contributes to the overall phase transition temperature of the membrane. The transition temperature is defined as the temperature at which the membrane changes from the liquid-crystalline to the gel state. Below this temperature there is a mixture of the two states. Above this temperature all of the membrane lipid is in the liquid-crystalline state. Because the plasma membrane structure of each cell type has a characteristic composition consisting of hetero-
geneous lipid components present in varying amounts, coexisting regions of different fluidity can occur in equilibrium within the same membrane (Thompson, 1980). Chilling a membrane can lead to a lateral migration of those lipid classes most readily gelled forming rigid assemblies capable of growing by the accretion of similar lipid species upon further chilling. This phenomenon is called phase separation, and leads to membranes containing regions of both liquid-crystalline and gel phases. Thus, the existence of possible phase separations in biological plasma membranes makes generalizations about membrane fluidity difficult. Nonetheless, measurement of membrane transition temperatures may provide some useful information regarding membrane dynamics.

The transition temperature of a membrane can be measured by a variety of techniques, each of which includes exposing the membrane to incremental increases or decreases in temperature while simultaneously monitoring intramembrane changes. One such technique is X-ray diffraction which detects alterations in the phase properties of the membrane bilayer based on intermolecular spacing between liquid-crystalline and gel phase lipids. This technique has been used to monitor the transition temperature in microsomal membranes from rat (Buhr et al., 1979) and bovine CL (Carlson et al., 1982). The X-ray diffraction pictures recorded at 39°C revealed two diffuse bands, one which represents membrane protein, and one
indicative of membrane phospholipid present in a liquid-crystalline state. Luteal membranes obtained from rats and cows during reproductive states corresponding to progesterone synthesis displayed transition temperatures of 37-39 C. Normally, the transition temperature for membrane lipids is below physiological temperature, which means that at body temperature the membranes are exclusively in the liquid-crystalline state. The appearance of gel phase lipid is indicated by a sharp band located peripherally to the band representing liquid-crystalline lipid. This sharp band was noted at 37-39 C in luteal membranes from rats and cows treated with PGF₂α, indicating that near body temperature, CL undergoing luteolysis had a portion of lipid in the gel phase. In addition, the transition temperature for CL membranes from the PGF₂α-treated animals was 46 C. The increased transition temperature indicates that a higher temperature is needed to "melt" the membrane, in other words the membrane contains more gel phase lipid. These results indicated that during periods of progesterone secretion, CL membranes consisted of liquid-crystalline phase lipid, but during luteolysis the appearance of the sharp band at body temperature indicated that the membrane contained gel phase lipid. A similar appearance of gel phase lipid has been observed in other cells undergoing deterioration. Thus physical alterations in CL membrane lipid resulted in decreased progesterone synthesis. Because progesterone
production in luteal cells depends upon LH, these researchers postulated that increases in gel phase lipid could curtail LH action at the plasma membrane.

According to the collision-coupling hypothesis of Levitzki (1978), the plasma membrane lipid milieu provides the matrix through which the hormone-receptor complex must migrate in order to interact with the Ns prior to activation of C. Based upon the kinetics of adenylate cyclase activation, Levitzki (1978) provided evidence to support this hypothesis that the hormone-receptor complex is not precoupled to Ns, does not dissociate from a precoupled state subsequent to hormone binding, but must transiently encounter Ns within the plasma membrane. This model predicts that alterations in membrane fluidity could affect hormone-stimulated adenylate cyclase activity.

Using turkey erythrocytes, Orly and Schramm (1975) demonstrated that the addition of cis-vaccenic acid to these cells enabled catecholamine-stimulation of adenylate cyclase to occur at 20 C. Adenylate cyclase was activated at 17 C in the presence of GMP-P(NH)P, but the addition of isoproterenol increased cyclase activity even more. These researchers suggested that cis-vaccenic acid facilitated hormone-induced activation of adenylate cyclase by causing increases in local membrane fluidity in the vicinity of the Ns. Rimon et al. (1978) provided direct evidence for the "fluidizing" effect of cis-vaccenic acid by simultaneously monitoring fluorescence polarization, a measure of
membrane fluidity, and catecholamine-induced cyclase activity. Subsequent experiments using this same cell system supported the importance of membrane fluidity in the activation of adenylate cyclase by hormones (Hanski et al., 1979; Briggs and Lefkowitz, 1980). These data indicate that an appropriate membrane environment is necessary for hormone-receptor interaction with Ns, and that increased membrane fluidity in the local environment of these membrane proteins resulted in maximal cyclase stimulation.

Membrane fluidity is a rather broad term that encompasses both the rate and extent of movement of lipids as well as integral membrane proteins. The types of fluidity that can occur within the lipid matrix of the membrane include lateral diffusion of the lipid within the monolayer, vertical diffusion across the bilayer, and rotational diffusion (Shinitzky, 1984). Lateral diffusion within the bulk of the lipid bilayer occurs fairly rapidly, and displays diffusion coefficients on the order of $10^{-9}$ to $10^{-7}$ cm$^2$ sec$^{-1}$ (Edidin, 1974). It has been estimated that each lipid molecule can exchange with its neighbor $10^6$ times per second, and that a lipid could theoretically diffuse from one side of a cell to another within a few minutes. Lateral diffusion of lipids is often complicated by the fact that most biological membranes are heterogeneous with respect to their lipid composition, such that the inner and outer bilayers can express asymmetric fluidity characteristics. For example,
the outer bilayer of erythrocytes was shown to be more fluid than the inner bilayer (Schachter et al., 1983). Vertical diffusion of lipids from the inner bilayer to the outer bilayer, sometimes referred to as membrane "flip-flop", occurs in biological membranes, but is rarely encountered in artificial membranes. The insertion of new lipids into membranes can produce asymmetrical stress on the inner bilayer leading to its collapse, thus driving the inner bilayer lipids into the outer bilayer. Vertical diffusion in some membrane systems is associated with methylation of PE to form PC and will be discussed in a later section. Rotational diffusion can occur about an axis perpendicular to the plane of the bilayer or transversely (Schinitzky, 1984).

The lateral movement of integral membrane proteins, such as hormone receptors, can be affected by many factors (for review, see Nicolson, 1979). Aggregation of proteins within the bilayer may restrict their movement based upon protein-protein interactions. A variety of studies have indicated that biological plasma membranes are not random mixtures of phospholipids, but can be divided into regions of a particular composition and order (Edidin, 1982; Edidin and Sessions, 1983). Such "domains" of membrane lipids could serve to sequester integral proteins into specialized regions of the cell, or exclude them from others. The presence of peripheral membrane components (proteins, carbohydrates), either at the cytoplasmic face
of the inner bilayer, or in the extracellular space exposed to the outer bilayer, could restrict the lateral movement of membrane proteins. Lastly, plasma membrane-associated cytoskeletal elements could either impede protein movement or facilitate protein translocation within the bilayer. The movement of proteins is much slower than the mobility of membrane lipids. The diffusion constants of proteins in biological membranes can range from $10^{-12}$ to $10^{-9}$ cm$^2$ sec$^{-1}$.

Thus, when discussing the general concept of "membrane fluidity", one must be cognizant that this cellular phenomenon encompasses many interactions involving both lipids and proteins, many of which may not be mutually exclusive.

A common method used to study the bulk fluidity of plasma membranes is fluorescence polarization (Shinitzky and Barenholz, 1978). This technique detects "membrane fluidity" by measuring the relative changes in the rotation time of a fluorescent probe under steady state conditions. Excitation of the probe is achieved by passing polarized light of a specified wavelength through the membrane sample, and simultaneously measuring the emission of light at a different wavelength, both parallel and perpendicular to the plane of excitation. Fluorescence polarization is determined by calculation of the ratios of parallel and perpendicular light emission, and is determined over a wide range of temperatures. A decrease in
polarization corresponds to increased movement in the lipid bilayer and thus reflects an increase in fluidity. Two commonly used lipid probes are diphenylhexatriene, which partitions equally into liquid-crystalline and gel phase lipids (Shinitzky and Barenholz, 1978), and pari-naric acid, a naturally occurring octadecatetraenoic acid, which preferentially partitions into gel phase lipids (Sklar et al., 1979). Incorporation of these probes into biological membranes does not alter cellular functions, and can thus be used as sensitive indicators of membrane changes without causing cellular side-effects.

A second technique actually measures the lateral diffusion of membrane lipids and proteins. This method is called fluorescence recovery after photobleaching (FRAP). A membrane of cell sample containing a fluorescent probe is exposed to a brief pulse of laser light which "bleaches" the membrane without causing permanent damage. The gradual recovery of fluorescence into the bleached area is measured with respect to its rate of recovery (diffusion constant) as well as the percentage of cells displaying recovery of the probe. A decrease in the diffusion constant and recovery of the probe indicate an increase in the viscosity of the membrane, if a lipid probe is used, or decreased lateral mobility if a protein probe is used.

These techniques have been utilized in a series of elegant experiments designed to study the characteristics
of rat and bovine CL during luteolysis. Microsomal and plasma membranes from rat CL during luteolysis displayed increased transition temperatures which were positively correlated to an increase in polarization ratios, indicating that the membranes were less fluid (Buhr et al., 1981; Carlson et al., 1984). In contrast, membranes obtained from luteal tissue that was actively secreting progesterone displayed lower polarization ratios indicative of fluidity. Parinaric acid appeared to be a better probe than diphenylhexatriene in elucidating changes in membrane fluidity of regressing rat CL (Carlson et al., 1984). The decrease in membrane fluidity during luteolysis did not appear to result from major changes in the fatty acid composition or cholesterol:phospholipid ratio of rat luteal membranes (Carlson et al., 1981). Membranes from bovine CL obtained during natural or PGF$_2$α-induced luteolysis also displayed increased transition temperatures (Carlson et al., 1982) and decreased bulk membrane fluidity (Goodsaid-Zalduondo et al., 1982) which correlates to the appearance of gel phase lipid during this reproductive stage. In contrast to the rat CL, an increase in SPH:PC was noted in regressing bovine CL suggesting the decreased membrane fluidity resulted from an increase in SPH. No changes in the proportions of total membrane lipid unsaturated or saturated fatty acids were observed (Goodsaid-Zalduondo et al., 1982). Taken collectively, these experiments indicated for the first
time that one of the mechanisms involved in the ability of PGF₂α to cause luteolysis may be linked to physical changes, i.e. decreased fluidity, of the luteal plasma membrane. Unfortunately, adenylate cyclase activity was not measured in these preparations, so one can only speculate as to the importance of membrane fluidity in LH-induced progesterone synthesis. In addition, it cannot be ascertained from these studies whether the decline in membrane fluidity was a direct cause of PGF₂α action on the luteal cell membrane or a consequence of chronic exposure to the luteolytic actions of PGF₂α. Nevertheless, these studies provide an important basis for further research regarding plasma membrane function in CL.

The addition of cholesterol to rat Leydig cells caused a decrease in membrane fluidity assessed by fluorescence depolarization that correlated with decreased cAMP and testosterone production (Kolena et al., 1986). The results were interpreted on the basis that cholesterol-enrichment of membranes already in a fluid state, as most are at body temperature, increased membrane rigidity. Cholesterol inhibition of Leydig cell responsiveness could possibly be due to a decrease in membrane fluidity which in turn results in a decreased interaction between adenylate cyclase and the hormone-receptor complex as has been observed in turkey erythrocytes (Rimon et al., 1978).

The lateral diffusion of the LH receptor in ovine luteal cells has been recently assessed using LH or hCG
conjugated to a fluorescent label (Niswender et al., 1985a). These hormone conjugates were biologically active with respect to LH receptor binding. Luteal cells containing the bound label were subjected to FRAP techniques. The oLH-LH receptor complex demonstrated a diffusion coefficient of $1.9 \times 10^{-10}$ cm$^2$ sec$^{-1}$, a value comparable to cell surface proteins labeled with concanavalin A. However, hCG-LH receptor complexes were immobile during the time scale of the experiment, thus displaying diffusion coefficients less than $10^{-11}$ cm$^2$ sec$^{-1}$. These data demonstrate that LH-receptor complexes can move laterally in the CL membrane, but that the sialic acid content of hCG may restrict the movement of the hCG-receptor complex.

The unoccupied LH receptor was also mobile in the ovine luteal cell membrane (Roess et al., 1986). Receptors occupied by hCG in preparations of luteal cell membranes that were free of underlying cytoskeletal components displayed lateral movement similar to that of LH-receptor complexes. These data suggest that in addition to sialic acid, the cytoskeleton associated with the plasma membrane may restrict the movement of hCG-occupied LH receptors. This phenomenon may be more important in determining the rate of internalization of the hormone-receptor complex, which is much slower for hCG- than LH-occupied receptors, rather than in LH- or hCG-induced progesterone synthesis, which is similar for both hormones. These data do not preclude the possible movement of gonadotropin receptor complexes.
in the activation of adenylate cyclase.

Using ferritin-LH conjugates bound to rat luteal LH receptors, it was shown that as more LH receptors became occupied, the receptors formed small microaggregates due to lateral movement in the membrane (Luborsky and Behrman, 1979). This lateral movement was reduced by PGF$_2$α at 37 C, but not at 4 C when membrane fluidity was reduced (Luborsky et al., 1984). These data provide circumstantial evidence to indicate PGF$_2$α may restrict lateral movement of LH-receptor complexes thus preventing coupling to adenylate cyclase.

In conclusion, the relationship between PGF$_2$α and reductions in plasma membrane fluidity of luteal cells provides a provocative basis for further studies regarding the mechanism of action of this hormone. Additional investigations regarding the physical properties of luteal plasma membranes will hopefully delineate whether increased membrane fluidity and/or lateral movement of the LH-receptor complex is required for activation of adenylate cyclase.

Hormonal Regulation of Phospholipid Methylation

Phosphatidylcholine (PC) is the major phospholipid of many endocrine tissues, including the corpus luteum (Scott et al., 1968), the bulk of which is synthesized in the cytidine diphospho-choline pathway (Thompson, 1980). A small proportion is also synthesized by the successive
enzymatic addition of three methyl groups to phosphatidyl-ethanolamine (PE) using S-adenosylmethionine (SAM) as the methyl donor (Hirata and Axelrod, 1978). A role for the methylation of PE has been implicated in the transduction of receptor-mediated signals through plasma membranes of a variety of cell types (Hirata and Axelrod, 1980).

The conversion of PE to PC is catalyzed by two membrane-bound enzymes that are asymmetrically distributed and characterized by different properties (Strittmatter et al., 1981). The first enzyme, phospholipid methyl-transferase I (PMT I), transfers one methyl group from SAM to PE to form phosphatidyl-N-monomethylethanolamine (PME). This enzyme has a high affinity for SAM ($K_m = 2 \ \mu M$), requires magnesium ion and is located facing the cytoplasmic side of the membrane bilayer. The sequential transfer of two additional methyl groups to PME to form PC is catalyzed by PMT II, which has a low affinity for SAM ($K_m = 100 \ \mu M$), does not require divalent cations and is localized on the outer surface of the membrane bilayer. As PE in the inner bilayer is being successively methylated, the two enzymes concomitantly facilitate its translocation across the membrane bilayer such that PC is subsequently located in the outer bilayer. The enzyme-mediated "flip-flop" movement of the methylated phospholipids occurs very rapidly (less than two minutes) in rat erythrocytes, which is in contrast to the spontaneous diffusion of phospholipids across artificial and natural
membrane liposomes which can take hours or days (Hirata and Axelrod, 1980).

The methylation and rapid asymmetric rearrangement of phospholipids has been shown to influence membrane fluidity by decreasing the microviscosity as measured by fluorescent polarization using the probe 1,6-diphenyl-1,3,5-hexatriene (Hirata et al., 1979). It appeared that the methylation of PE, the rapid transfer of monomethylated PE or both resulted in the increased membrane fluidity, whereas methylation to PC had little effect on membrane viscosity.

The biological importance of the phospholipid methylation reaction was first examined by studying the β-adrenergic receptor/adenylate cyclase system of rat reticulocytes in a series of experiments summarized by Strittmatter et al. (1981). It was observed that incubation of rat reticulocytes with SAM and β-adrenergic agonists increased the rate of phospholipid methylation, which was reflected by increases in both PMT I and II activities and increased PC synthesis. This stimulation of phospholipid methylation appeared to be mediated by agonist-occupation of the β-adrenergic receptor because the ability of various agonists to stimulate methylation matched their ability to bind to the receptor. The binding of β-antagonist inhibited methylation, and the addition of fluoride ion or cholera toxin (compounds which activate adenylate cyclase without involving the β recep-
tors) or dibutyryl cAMP had no effect on methylation.

In view of the observations that phospholipid methylation increased membrane fluidity (Hirata et al., 1979), and that lateral movement of the β-adrenergic receptor facilitated by this increased fluidity can increase the coupling between the receptor and adenylate cyclase (Rimon et al., 1978), the possible role of methylation in enhancing β-agonist-induced cyclase activity was examined. Indeed, the presence of SAM significantly enhanced isoproterenol-induced cyclase activity at a concentration which caused activation of PMT I. Thus, Hirata and Axelrod (1980) proposed that agonist binding to receptor stimulates phospholipid methylation which in turn increases membrane fluidity in a local area such that coupling between the ligand-receptor complex and the guanine nucleotide regulatory subunit of adenylate cyclase by lateral diffusion is enhanced leading to the generation of cAMP and specific cellular events. Although Vance and de Kruijff (1980) have argued that the magnitude of PE methylation in response to agonists is far too small to evoke any functional changes in the plasma membrane, Hirata and Axelrod (1980) provide fairly convincing evidence to suggest that small changes in phospholipids within local membrane domains are sufficient to induce perturbations that lead to important agonist-induced cellular responses.

The relationship between phospholipid methylation and
LH-induced progesterone synthesis by bovine luteal cells in vitro has been recently examined by Milvae et al. (1983). The addition of two different methylation inhibitors, S-adenosylhomocysteine and 3-deazaadenosine, did not affect progesterone production when given alone, but caused a significant reduction in steroidogenesis induced by LH. When SAM plus LH were present in the incubation media, progesterone production was stimulated above that obtained with LH alone, while SAM was without effect in the absence of LH. These authors also reported that preliminary experiments revealed the incorporation of methyl groups into PME and PC only when LH was present. It is difficult to assign a subcellular localization for phospholipid methylation in the study of Milvae et al. (1983) because the effects of SAM were monitored in whole luteal cells. In an attempt to suggest a plasma membrane location for the methylation reaction, these researchers demonstrated that S-adenosylhomocysteine did not inhibit cholera toxin- or dibutyryl cAMP-induced progesterone synthesis, indicating the inhibitory effect of this metabolite occurred at a site prior to the activation of adenylate cyclase. Therefore, the mechanism by which phospholipid methylation increases the ability of LH to induce progesterone synthesis and whether this is a plasma membrane phenomenon involved in the activation of adenylate cyclase in the luteal cell remain to be clarified.
Two recent investigations also suggest a role for phospholipid methylation by glycoprotein hormones. Stimulation of rat Leydig cells (Nieto and Catt, 1983) and thyroid glands (Prasad and Edwards, 1984) with hCG and TSH, respectively, resulted in a time- and dose-dependent increase in PMT activity, incorporation of tritiated methyl groups in PC and PME, and increased testosterone or thyroxine secretion. In contrast to the primary role of phospholipid methylation in receptor-cyclase coupling assigned by Hirata and Axelrod (1980), PMT activity in Leydig cells was stimulated by 8-bromo-cAMP or cholera toxin plus phosphodiesterase inhibitor, and 8-bromo-cAMP also increase PME synthesis (Nieto and Catt, 1983). These results suggest that phospholipid methylation in hCG-stimulated Leydig cells is involved in membrane events subsequent to cAMP production rather than enhancing receptor-cyclase coupling. It is possible that phospholipid methylation may be related to the transient increase in LH receptors observed within the first few hours after in vivo LH treatment in rat Leydig cells (Huhtaniemi et al., 1981) and ovine luteal cells (Suter et al., 1980). Increased membrane fluidity enhanced by phospholipid methylation was accompanied by increased receptor binding of isoproterenol in rat reticulocytes (Strittmatter et al., 1979) and growth hormone in mouse mammary glands (Bhattacharya and Vonderhaar, 1979). De novo protein synthesis was not possible in these membrane preparations, thus it
was suggested that the appearance of new receptor sites may involve changes in membrane microviscosity, thereby unmasking "cryptic" receptors (Strittmatter et al., 1979). Increasing membrane fluidity of primate CL by treatment with ethanol and neuraminidase also exposed masked LH receptors (Danforth and Stouffer, 1985). A direct role for phospholipid methylation in TSH-induced adenylate cyclase activity leading to thyroxine secretion is not indicated because inhibitors of methylation that decrease PC formation did not affect secretion, suggesting the involvement of this reaction in other thyroid cell functions (Prasad and Edwards, 1984). Thus, the precise physiological significance of glycoprotein hormone-stimulated phospholipid methylation, particularly as it relates to membrane processes, remains to be demonstrated. Phospholipid methylation does, however, pose an interesting possibility as a common mechanism whereby many receptor-mediated signals are transmitted through plasma membranes.

Ganglioside-Glycoprotein Hormone Interactions

Gangliosides comprise a family of acidic glycolipids, containing both polar and nonpolar components, characterized by the presence of sialic acid (Fishman and Brady, 1976). Gangliosides are primarily localized in membranes and are enriched in plasma membranes. By nature of their structure, it is presumed the orientation of gangliosides within membranes is such that the hydrophobic moiety,
ceramide, is buried in the lipid bilayer while the hydrophilic oligosaccharide chain is exposed at the cell surface where it is potentially available to interact with biological effectors of cell function whose actions are initiated at the plasma membrane. The negatively-charged carbohydrate portion consists of sialic acid, hexoses and N-acetyl hexosamines in various combinations according to the ganglioside species, linked through a glycosidic bond to the sphingosine portion of ceramide. The sequential addition of monosaccharide residues to ceramide is catalyzed by highly specific glycosyltranferase enzymes whose presence within cells dictates and is reflected by the ganglioside composition of the cell. Brain and neural tissues have the greatest quantities of lipid-bound sialic acid represented by a complex ganglioside pattern, while most extraneural tissues lack specific glycosyltransferases and thus, their respective products, gangliosides. However, bovine (Mullin et al., 1976) and rat (Meldolesi et al., 1976) thyroid gland membranes as well as rat testicular membranes (Lee et al., 1977) have been shown to contain substantial quantities of higher-order gangliosides not previously observed in extraneural tissues.

The importance of gangliosides in the transmission of membrane-mediated signals necessary for cellular function was evident when the ganglioside, GM₁, was discovered to be a specific component of the receptor for cholera toxin (see reviews by Fishman and Brady, 1976; Kohn, 1978).
Each cholera toxin molecule is composed of five covalently linked, identical β subunits and one A subunit that consists of two nonidentical peptides, linked by disulfide bridges, called A₁ and A₂. Following binding of the β subunits to the "GM₁ receptors", the toxin molecule undergoes a conformational change that allows the A subunit to dissociate from the B protein. As the A subunit translocates within the lipid bilayer of the plasma membrane, the disulfide bonds are cleaved forming an activated fragment, A₁, which can stimulate adenylate cyclase activity by ADP-ribosylation of the guanine nucleotide regulatory subunit as described previously.

In light of the currently accepted mechanism of action of cholera toxin, Kohn (1978) proposed the possibility that glycoprotein hormones might utilize a similar sequence of membrane events leading to stimulation of adenylate cyclase. In a series of studies involving TSH-receptor interactions and summarized below, Kohn (1978) presented evidence to support this hypothesis by demonstrating that gangliosides may be a component of the glycoprotein hormone receptor and that peptide sequence homologies exist within the α and A subunits as well as the β and B subunits of glycoprotein hormones and cholera toxin, respectively, suggesting analogous modes of action.

A variety of gangliosides were evaluated for their effects on the binding of TSH to bovine thyroid receptors (Mullin et al., 1976). Gangliosides inhibited the binding
of $^{125}$Iiodo-TSH to thyroid plasma membranes, and this inhibition was critically related to the number and location of sialic acid residues within the ganglioside structure. The most efficacious inhibitor was $G_{D1b}$, a ganglioside consisting of two sialic acids bound to the internal galactose residue of the oligosaccharide chain. The addition of sialic acid to the terminal galactose, as in $G_{T1}$, reduced the binding inhibition only slightly. If one of the internal sialic acids was deleted, as in $G_{D1a}$, no binding inhibition was observed. The cholera toxin receptor $G_{M1}$, which contains an internal sialic acid, was intermediate in this ability to inhibit TSH binding.

Similar studies showed that gangliosides also inhibited the binding of labeled LH to rat testes membranes, the efficacy of inhibition from greatest to least being:

$G_{T1} > G_{D1b} > G_{D1a} > G_{M1} > G_{M2}$ (Lee et al., 1977). The pattern of inhibitory potency of the gangliosides for LH binding was distinct from that of TSH ($G_{D1b} > G_{T1} > G_{M1} > G_{M2} > G_{M3} > G_{D1a}$) as well as hCG ($G_{T1} > G_{D1a} > G_{D1b} > G_{M2} > G_{M1}$; Lee et al., 1976). These results suggest that if gangliosides are components of the glycoprotein hormone receptors, the ganglioside structure is an important determinant of the specificity of the hormone interaction with its respective target cell. Neither sialic acid alone, nor fetuin, a sialic acid-containing protein, affected TSH binding suggesting that the structure of the entire oligosaccharide chain is important in the inter-
action. It was also evident that insulin, glucagon, prolactin, growth hormone, FSH and adrenocorticotropic (ACTH) could not prevent the ganglioside inhibition of TSH binding, indicating hormonal specificity of this interaction (Mullin et al., 1976).

When membranes are preincubated with gangliosides prior to their exposure to labeled TSH (Mullin et al., 1976), LH (Lee et al., 1977) or hCG (Lee et al., 1976), no inhibition of hormone binding was observed, thus providing evidence that the gangliosides are interacting specifically with the hormones themselves and not with the target cell membranes. The ultracentrifugation and chromatographic elution patterns of TSH obtained in the presence and absence of gangliosides were different, which indicated the formation of TSH-ganglioside complexes (Mullin et al., 1976).

If glycoprotein hormone interaction with gangliosides is analogous to that of cholera toxin with G\textsubscript{M1}, gangliosides should induce a conformational change in the hormone. This was indeed observed for TSH (Mullin et al., 1976), LH (Lee et al., 1977) and hCG (Lee et al., 1978) by measuring the increase or decrease in tyrosine fluorescence of the hormone in the presence of the various gangliosides. The pattern of changes in fluorescence corresponded to the degree of inhibition of hormone binding induced by the ganglioside, thus G\textsubscript{D1b} and G\textsubscript{T1} decreased the fluorescence of TSH and LH/hCG tyrosine
residues, respectively. The precise structural nature of the ganglioside-TSH interaction is unknown, but an additional study utilizing dansylated TSH, which retained receptor binding activity and its ability to activate adenylate cyclase, suggested that electrostatic interactions such as hydrogen bonding of the glycerol moiety of the sialic acid residue to amino acid residues of TSH were initially involved because calcium ion could not disrupt a preformed ganglioside adduct, but was effective in disrupting TSH-phospholipid adducts (Aloj et al., 1979). Thus, these researchers speculated that the ganglioside participates initially in electrostatic interactions with TSH, which may be supplemented by short-range hydrophobic interactions. This possibly results in the exclusion of salts and water from the adduct such that a portion of the "properly oriented" TSH molecule is capable of entering the lipid bilayer and affecting adenylate cyclase or other membrane functions.

Additional evidence to support the proposition that glycoprotein hormones and cholera toxin may have a similar mechanism of action is indicated by sequence homologies in the β and B chains, respectively (Kohn, 1978). A computer search of the peptide amino acid sequences revealed that a CAGY (cysteine-alanine-glycine-tyrosine) region in the β chains of the glycoprotein family (TSH residues 27-30, LH/hCG residues 34-37, FSH residues 32-35) was homologous to a CAGY (cysteine-alanine-glutamic acid-tyrosine)
sequence in residues 9-12 of cholera toxin. That the CAGY sequence is highly conserved implies the existence of peptide regions critical for biological function and not susceptible to mutation. Thus, this homologous sequence may represent an important locus involved in the binding of toxin and hormone to their receptors because the B or β subunits are necessary for binding as well as lending target organ specificity.

A structure-function analogy has also been extended to include the A and α subunits (Kohn, 1978) in that a more restricted area of sequence homology has been identified. Although no evidence exists to demonstrate that the α subunits of glycoprotein hormones are directly capable of stimulating adenylate cyclase activity, this sequence homology suggests a role for this peptide in comparison to the A₁ subunit of cholera toxin. Even more striking, however, was the observation of a larger amino acid sequence homology (six of nine residues) among the α subunits of glycoprotein hormones and the nonapeptide neurohypophyseal hormones oxytocin, arginine vasotocin, arginine vasopressin and lysine vasopressin, whose action presumably also involves adenylate cyclase stimulation. The primary functions of these nonapeptides include glandular or muscle contraction, diuretic and vascular effects that are manifested through changes in electro-chemical gradients and membrane ion transport. This suggests the possibility that the α subunits of glyco-
protein hormones may not only modify cellular events by increasing intracellular cAMP levels, but also may alter membrane transport events as well.

A number of criticisms of Kohn's theory have emerged. Studies using more physiological concentrations of gangliosides have shown that the binding of hCG to rat testes preparations is not inhibited by gangliosides (Pacuszka et al., 1978; Ahzar and Menon, 1979). Acidic phospholipids, such as PI, were found to be more potent inhibitors of TSH binding to bovine thyroid membranes as compared with gangliosides (Omodeo-Sale et al., 1978). However, research from Kohn's laboratory also indicated a direct interaction between PI and TSH in solution, but not when PI is embedded in a liposome, whereas gangliosides were effective in both preparations (Aloj et al., 1979). An additional criticism is a complete lack of evidence for a direct interaction of the α subunits of glycoprotein hormones with either the regulatory or catalytic components of adenylate cyclase. Pierce and Parsons (1981) provide evidence to suggest that the CAGY sequence of the β subunits is a region of subunit-subunit contact within glycoprotein hormones, and thus would not be accessible for the interaction with the receptors and(or) cyclase. Pierce and Parsons (1981) also state that preparations of LH in which the α and β subunits are crosslinked retain both receptor binding and biological activities, suggesting that the α subunit need not dissociate from the β
subunit to elicit target cell responses.

A recent model to explain the hormone-receptor interaction of the α and β subunits of hCG put forward by Milius et al. (1983) bears a resemblance to that proposed by Kohn. Milius et al. (1983) observed that the binding ability of various polyclonal antisera specific for the β subunit was unaffected when the antiserum was incubated with solubilized hCG-receptor complexes. In contrast, α subunit-specific antisera were unable to bind to hCG once it was associated with the receptor. These results suggested that the β subunit is not directly involved with the receptor in the final state of the hormone-receptor complex, whereas the α subunit directly interacts with the receptor or an adjacent portion of the plasma membrane because the immunoreactive sites were masked. These immunoreactive sites on the α subunit may undergo a conformation change as a result of receptor binding such that antisera binding is inhibited. These researchers proposed, then, that the initial step in the binding of hCG to the receptor involves a highly specific, but low affinity binding of the β subunit. This is followed by an "activation" of a second receptor site responsible for the high affinity binding of the α subunit. Thus, the α subunit, which is targeted to the luteal cell by the specificity of the β subunit binding, may then "have some common function among the glycoprotein hormones such as adenylate cyclase activation" (Milius et al., 1983). It
may be interesting to determine whether gangliosides are involved in this proposed conformation change of the \( \alpha \) subunit, or the \( \alpha \) subunit-receptor complex as was proposed by Kohn (1978). To date, there are no reports of the ability of gangliosides to interact with isolated \( \alpha \) and \( \beta \) subunits of glycoprotein hormones.

Although there appear to be valid criticisms pertaining to the role of plasma membrane gangliosides in the mechanism by which glycoprotein hormones may stimulate adenylate cyclase activity, the possibilities remain intriguing nonetheless, particularly in view of the fact that gonadotropin recombinants lacking carbohydrate in their \( \alpha \) subunits fail to activate adenylate cyclase (Sairam, 1983). Reevaluation of ganglioside interactions with purified glycoprotein subunits and the inclusion of their effects on adenylate cyclase activity in natural or reconstituted membrane systems may clarify the proposed functions of these glycolipids in biological membranes.

The Polyphosphoinositide Cycle

Research conducted over the past decade has contributed enormously to our understanding about the nature of the second messengers used by ligands that act on the plasma membrane to bring about their cellular responses through inositol lipid metabolism. Excellent reviews concerning membrane signal transduction and the polyphosphoinositide cycle have been provided by Berridge (1984;
One of the major events induced by agonist-dependent phosphoinositide metabolism is the hydrolysis of plasma membrane PI. Approximately 98% of the total inositol lipids in a cell consist of PI, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-diphosphate (PIP2), with PIP and PIP2 comprising only 2% of the total phosphatidylinositol lipids. A portion of these phospholipids exist in a hormone sensitive pool. In the absence of hormone-binding, levels of these phospholipids are maintained in a dynamic equilibrium by very active kinases and phosphomonoesterases, such that a change in the level of one is usually buffered by its interconversion to the others. This "futile" cycle is metabolically expensive because it requires high levels of ATP. The occupation of the hormone receptor by an agonist diverts PIP2 out of the futile cycle towards a membrane phospholipase C (also referred to as phosphodiesterase) that cleaves PIP2 to inositol 1,4,5-triphosphate (IP3) plus diacylglycerol (DG). Recent evidence suggests that the hormone-receptor complex is coupled to phospholipase C by a distinct GTP regulatory protein analogous to the Ns involved in adenylate cyclase activation (Cockcroft and Gomperts, 1985). Diacylglycerol remains associated with the inner
bilayer of the plasma membrane, while IP$_2$ is released into the cytosol. The sequence of events following agonist-induced hydrolysis occur within a matter of seconds and are reflected by a sharp decline in the level of PIP$_2$, a transient increase in DG, no change in the level of PI due to the large quantity of this phospholipid in relation to PIP$_2$, with a subsequent increase minutes post-hormone treatment in PIP$_2$ and phosphatidic acid (PA). Increases in intracellular IP$_2$ are seen concomitant with decreases in PIP$_2$. The IP$_2$ acts as a second messenger within the cell by causing the mobilization of intracellular calcium from the endoplasmic reticulum. An elevation in intracellular calcium levels is one mechanism whereby cellular responses are activated. Because most cells possess a calcium pump on their plasma membranes, it is possible, although not yet proven, that the changes in PIP$_2$ may regulate membrane fluidity (Sheetz et al., 1983) that could lead to inhibition of the calcium pump (Lin et al., 1983), or the opening of calcium channels. Cessation of the transient increase in IP$_2$, results from its rapid conversion to IP$_2$. Dephosphorylation of IP$_2$ to IP follows, and IP is finally converted to free inositol. Simultaneous conversion of DG to PA occurs, and free inositol can then combine with DG to result in PI, thus completing the cycle.

Exposure of rat (Leung et al., 1986) and bovine (West et al., 1986) luteal cells to PGF$_2\alpha$ in vitro resulted in
the rapid decrease in PIP$_2$ and concomitant increases in IP$_3$, IP$_2$, and IP. These are the first reports to demonstrate that the mechanism of action of PGF$_{2\alpha}$ binding to its receptor activates the phosphoinositide hydrolysis pathway, presumably leading to increases in intracellular calcium in luteal cells. Dorflinger et al. (1984) examined the effects of calcium on LH-induced adenylate cyclase activity in rat luteal cells. Their studies indicated that the calcium ionophore, A23187, inhibited LH-induced cAMP accumulation only in the presence of extracellular calcium. Depletion of extracellular calcium did not result in an inhibition of LH-induced cAMP accumulation by PGF$_{2\alpha}$. Verapamil, a calcium channel blocker, had no effect on PGF$_{2\alpha}$ inhibition of LH-induced cyclase activity. Incubation of isolated rat luteal membranes in the presence of 5-20 μM calcium produced a dose-dependent decrease in LH-stimulated adenylate cyclase activity. Collectively, these observations suggested that acute increases in intracellular calcium inhibits the activation of adenylate cyclase similar to the inhibition produced by PGF$_{2\alpha}$. In addition, the inhibition of cyclase activity did not depend upon an influx of extracellular calcium. The results of Dorflinger et al. (1984) are consistent with the current reports of PGF$_{2\alpha}$-induced increases in IP$_3$ accumulation in luteal cells, which may reflect increases in intracellular calcium mobilization. An exhaustive study on the properties of a calcium-magnesium ATPase in
rat luteal membranes revealed that it functions as a calcium-extrusion pump (Verma and Penniston, 1981). Prostaglandin F$_2$α-treatment of rats containing functional CL caused a dramatic reduction in calcium-ATPase activity within 1 to 2 hours (Alberts et al., 1984). Thus, the acute response of luteal cells to PGF$_2$α involves the hydrolysis of PIP$_2$ to IP$_3$, increased mobilization of intracellular calcium and the maintenance of high intracellular calcium levels which results in the inhibition of adenylate cyclase activity.

An important consequence of elevated intracellular calcium levels in luteal cells could be the activation of phospholipase A$_2$, an enzyme known to catalyze the release of arachidonic acid from the sn-2-position of the phospholipid glycerol moiety (Van den Bosch, 1980). Phosphatidylcholine, PE and PI contain fairly large quantities of esterified arachidonic acid. Riley and Carlson (1985) observed that the rapid rigidification of luteal plasma membranes from PGF$_2$α-treated rats was calcium dependent. Membranes from non-regressed CL did not show an increase in fluorescence polarization indicating that these membranes were fluid. However, exposure of membranes from non-regressed CL to phospholipase A$_2$ caused virtually identical changes in the polarization properties as in membranes obtained from regressing CL. The addition of a nonspecific phospholipase A$_2$ inhibitor, P-bromo-phenacyl-bromide, to membranes from regressed CL prevented the
decrease in membrane fluidity normally observed during luteolysis. These researchers suggested that increased endogenous phospholipase A₂ activity could cause degenerative changes in plasma membranes resulting in luteal regression. They proposed that phospholipase A₂ activity could lead to: 1) an elevation in luteal prostaglandin synthesis which could further accelerate regression in a positive feedback manner and 2) production of superoxide anion, through the action of lipoxygenases that use arachidonic acid as a substrate, which has been shown to cause degenerative changes in other cellular membranes. These researchers extended their observations using rat CL membranes labeled in vitro with 1-stearoyl-2-¹⁴C-arachidonoyl-PC (Riley and Carlson, 1986). Labeled free arachidonic acid and lyso PC accumulated in the media from labeled membranes obtained from regressing CL to a greater extent than in membranes from functional CL, suggesting the presence of an active phospholipase A₂ during luteolysis. Bovine, porcine and ovine CL contain substantial quantities of arachidonic acid predominantly esterified to phospholipids (Lukazewska and Hansel, 1980; Waterman, 1980a, 1981). Intracellular free arachidonic acid concentrations are very small (Lands, 1979; Waterman, 1980a,b), thus phospholipids and triglycerides serve as the main reservoirs of this substrate for prostaglandin synthesis. Bovine CL (Shemesh and Hansel, 1975) and porcine CL (Waterman, 1980b) are capable of producing PGF₂α in vitro.
Therefore, it is possible that intraluteal prostaglandin synthesis could occur in response to PGF$_2$$\alpha$-induced increases in intracellular calcium activating phospholipase A$_2$, although this remains to be directly demonstrated.

Tissues responsive to polypeptide hormones have also been shown to convert arachidonic acid to prostaglandins in the presence of high intracellular calcium concentrations. It is not evident from the studies on ACTH- or TSH-induced prostaglandin synthesis in the adrenal cortex (Laychock et al., 1977; Schrey and Rubin, 1979) or thyroid (Haye et al., 1976; Haye and Jacquemin, 1977), respectively, whether this was linked to polyphosphoinositide hydrolysis, but the activation of phospholipase A$_2$ appeared to be involved (Laychock and Putney, 1982). It is also not known whether LH is capable of stimulating arachidonic acid release from phospholipids in luteal tissue of domestic animals; however, Milvae and Hansel (1983) found that LH had no effect on bovine luteal PGF$_2$$\alpha$ or prostacyclin production in vitro on days 5 through 18 of the estrous cycle. Thus, it appears that if intraluteal prostaglandin synthesis occurs, it would most likely be a result of PGF$_2$$\alpha$- rather than LH-induced effects.

Polyphosphoinositide hydrolysis in some cells is also associated with arachidonic acid formation from DG and/or PA as a result of diacylglycerol lipase and a PA-specific
phospholipase A$_2$. This release of arachidonic acid initiated by calcium-mobilizing receptors appears to be distinct from the pathway deriving arachidonic acid from the other membrane phospholipids by the activation of less-specific phospholipase A$_2$. This latter pathway requires much higher levels of intracellular calcium than are provided by the transient increases in IP$_3$ formation, and may only occur under supramaximal stimulation by agonists or by mechanisms that maintain intracellular calcium at high, sustained levels.

The transient presence of DG in membranes as a result of hormone-induced polyphosphoinositide hydrolysis also plays an important role in generating cellular responses (Nishizuka, 1984; Nishizuka et al., 1984). Many tissues that are unresponsive to cAMP-dependent protein kinase activation, possess another kinase called protein kinase C (PKC). This enzyme has a $M_r = 77,000$ and consists of a hydrophobic domain which appears to bind to the inner bilayer of the plasma membrane, and a hydrophilic domain which contains the catalytically active site. During its resting state, PKC is loosely associated to the plasma membrane, but upon activation becomes more tightly coupled to it. The DG increases the affinity of PKC for calcium and PS which are required for kinase activation to occur. The effect of DG appears to be a result of the presence of unsaturated fatty acids that facilitate conformational changes in PKC enabling it to bind calcium. In a number
of cells, PKC has been shown to phosphorylate a 40,000 MW protein of unknown function, as well as a 20,000 MW protein identified as myosin light chain kinase. A class of tumor promoters, known as phorbol esters, can substitute for DG and activate PKC. Phorbol esters intercalate into the plasma membrane and bind with high affinity to PKC. This binding induces a conformational change in the membrane, with a subsequent change in the orientation of PKC so that its affinity for calcium is increased, resulting in activation. Thus activation of PKC results in the phosphorylation of proteins important for cellular function.

A protein kinase was isolated from bovine luteal cytosol, and shown to be dependent upon calcium and phospholipid for its activity (Davis and Clark, 1983). Brunswig et al. (1986) recently demonstrated that bovine luteal PKC could be activated by phorbol esters. Maximal stimulation of PKC could be obtained with 10 nM of the phorbol ester and resulted in increased progesterone production. Higher concentrations led to a decline in steroidogenesis. Phorbol ester-induced progesterone production was not accompanied by increases in cAMP, but was greater in the presence of LH indicating an additive effect. The conversion of 25-hydroxycholesterol to pregnenolone was not affected by phorbol ester, suggesting its site of action was between the formation of cAMP and the formation of pregnenolone in the mitochondria. It is
interesting to note that these researchers used small bovine luteal cells in their experiments. This activation of PKC in large luteal cells has not been assessed, but may prove to be an interesting mechanism to study because large cells from ovine CL produce progesterone at a maximal rate without the activation of cAMP-dependent protein kinase (Hoyer and Niswender, 1985). The endogenous agent responsible for PKC activation in luteal cells is not known, and the substrates for phosphorylation by PKC remain to be identified.

Steroidogenic agents, such as ACTH and LH, have also been found to provoke changes in polyphosphoinositide metabolism (for review, see Farese, 1983a,b). Although studies indicated a role for ACTH in phospholipase A2 activation, the hydrolysis of plasma membrane polyphosphoinositides has not been observed in the action of ACTH-induced steroidogenesis. Early efforts attempted to identify an ACTH-induced, cyclohexamide sensitive factor present in adrenal cytosols that enhanced the association of cholesterol to side chain cleavage activity when added to adrenal mitochondria. It was expected that this factor would be a protein, but it could not be identified by Farese (1983b). Rather, PIP2, PIP3, and cardiolipin were present in the stimulatory cytosol fractions, and it was shown that these phospholipids were effective in promoting side chain cleavage activity. Subsequent studies conducted to evaluate the effects of ACTH on adrenal phos-
phospholipid production revealed that ACTH-induced increases in DPI and TPI were preceded by rapid, small increases in PA and PI, and were accompanied by small increases in DG. Increases in PC and PE were also induced by ACTH. Farese (1983a,b) concluded that ACTH primarily increases de novo phosphatidate synthesis, and this leads to a generalized increase in phospholipid synthesis. These changes in phospholipids closely paralleled the time course for steroidogenesis in adrenal tissue. The levels of phospholipids were also increased by cAMP, suggesting that this effect of ACTH is mediated by its second messenger. Calcium and protein synthesis were also required for increased phospholipid synthesis, which is of interest because these requirements are also necessary for steroidogenesis. The addition of PIP and PIP₂ to rat adrenal cells in vitro increased steroidogenesis. Although ACTH-induced increases in PIP₂ and PIP occurred within minutes, this presumably reflects the fact that the basal levels of these phosphoinositides is generally low, thus they have a high rate of turnover. The ACTH-induced increases in phosphoinositides and phospholipids were not due to PIP₂ hydrolysis because this effect has not been demonstrated in adrenal cells, and is not sensitive to cyclohexamide.

Davis et al. (1981) observed that LH increased total levels of bovine luteal phospholipids in vitro within 30 minutes (the earliest time studied). Total phospholipid synthesis was evident at 120 minutes post-LH, and was
reflected by major increases in PA, PI, PC, PE and small increases in PIP and PIP₂. The increases in phospholipids paralleled LH-induced progesterone production as well. Similar observations were made in LH-treated rat Leydig cells (Lowitt et al., 1982). In rat granulosa cells, LH induced increases in PA within 2 minutes and PI within 10 minutes, which were sustained for 60 minutes and paralleled progesterone synthesis. Bovine luteal and rat granulosa cells do not require cAMP as an intermediate in LH-induced phospholipid synthesis, but a cyclic AMP analog produced a similar increase in phospholipid synthesis as observed with LH in rat Leydig cells.

Because the effects of phospholipid metabolism were measured in whole luteal cells and paralleled progesterone synthesis in vitro, the de novo phosphatidate pathway is most likely involved in the action of LH on steroidogenesis, similar to that observed in ACTH action on adrenal cells. It is not known if the LH-induced increases in phospholipids play a role in plasma membrane function, but it appears they are most likely more important for mitochondrial steroidogenesis.

In summary, polyphosphoinositide metabolism appears to play an important role in luteal function. Hydrolysis of PIP₂ to IP₃, the mobilization of intracellular calcium, and the attenuation of calcium transport out of the cell are mediated by PGF₂α. The generation of DG by PIP₂ hydrolysis also activates PKC in luteal cells, but its
regulation and function is unknown. De novo synthesis of phospholipids by LH appears to play an important role in steroidogenesis.

**Interactions Between LH and PGF$_2$α at the Luteal Cell**

This final section will be an attempt to summarize the cellular mechanisms underlying the regulation of luteal function by PGF$_2$α and its possible points of interaction with LH at the level of the luteal cell. The following discussion will particularly focus on the events that occur at the luteal cell plasma membrane, and will include what is currently known with respect to rat, ovine and bovine CL. This cumulative sequence of events is speculative, but provides a framework with which to organize the available observations.

The *in vivo* administration of PGF$_2$α to ewes on day 9 of the estrous cycle caused a decline in serum progesterone between 4 and 7.5 hours post-injection (Diekman et al., 1978b; Agudo et al., 1984). The decline in luteal progesterone content was not observed until 12 to 24 hours post-PGF$_2$α. Nett and Niswender (1981) reported a decrease in luteal blood flow within 2 hours following intrauterine administration of PGF$_2$α, thus decreases in plasma progesterone may be expected to occur prior to decreases in luteal progesterone content. Basal and LH-
stimulated adenylate cyclase activity also decreased in CL 2 hours after PGF₂α, and continued to decline over the following 22 hours (Agudo et al., 1984). An effect of PGF₂α on blood flow to the CL cannot be discounted as a significant mechanism by which it induces luteolysis. However, exposure of rat CL to PGF₂α in vitro resulted in a decrease in LH-stimulated cAMP accumulation within 15 minutes (Lahav et al., 1976). Similarly, Fletcher and Niswender (1982) reported that ovine luteal cells in vitro had to be exposed to PGF₂α for at least one hour before decreases in LH-stimulated adenylate cyclase activity were observed.

The decline in LH-induced adenylate cyclase activity by PGF₂α does not appear to result from a decrease in LH receptor concentrations. Serum progesterone concentrations had declined before decreases in either occupied or unoccupied receptors were detected, the latter decrease occurring at 22.5 hours post-PGF₂α (Diekman et al., 1978b). The inhibition of LH-induced cAMP accumulation that occurred within one hour in rat luteal cells was also not accompanied by decreases in LH receptor binding (Thomas et al., 1978).

Specific receptors for PGF₂α have been found in ovine (Powell et al., 1974; Fitz et al., 1982) and bovine (Powell et al., 1976; Lin and Rao, 1977) luteal cell plasma membranes. Concentrations of receptors in bovine CL were elevated during the luteal phase of the estrous
cyclé (Bartol et al., 1981). Thus, it is assumed that PGF$_2\alpha$ acts by initially binding to a plasma membrane receptor. Upon binding, the PGF$_2\alpha$-receptor hormone complex activates the hydrolysis of PIP$_2$ to IP$_3$ and DG (Leung et al., 1986; West et al., 1986). Inositol triphosphate acts as a second messenger and causes the mobilization of intracellular calcium from the endoplasmic reticulum (Berridge, 1984). Elevations of intracellular calcium in luteal cells is presumably not transient, but sustained because PGF$_2\alpha$ also appears to attenuate the activity of calcium pump located in the luteal cell plasma membrane (Verma and Penniston, 1981; Alberts et al., 1984). Elevated levels of intracellular calcium inhibit LH-stimulated adenylate cyclase activity and progesterone synthesis (Dorflinger et al., 1984).

The effects of elevated intracellular calcium levels in luteal cells could be manifested in many ways. Agudo et al. (1984) observed an increase in phosphodiesterase activity as early as 2 hours post-PGF$_2\alpha$. This enzyme requires calcium (Beavo et al., 1982), and catalyzes the conversion of CAMP to 5'-AMP, thus decreasing the concentration of CAMP available to activate CAMP-dependent protein kinase. Phospholipase A$_2$ also requires high levels of calcium, and the activation of this enzyme in rat luteal plasma membranes has been associated with decreased membrane fluidity (Riley and Carlson, 1985). Decreased membrane fluidity could prevent the lateral
diffusion of the LH-receptor complex and the $N_S - C$ within the plane of the membrane, thus preventing stimulation of adenylate cyclase. Cholera toxin- and NaF-stimulated cyclase activity in PGF$_2\alpha$-treated ovine luteal cells was not impaired, suggesting the effect of PGF$_2\alpha$ was in the coupling of the LH receptor to $N_S$ (Fletcher et al., 1982). However, Agudo et al. (1984) observed that GMP-P(NH)$_2$P-stimulated adenylate cyclase activity decreased in parallel with basal adenylate cyclase activity over 24 hours in ovine luteal cells exposed to PGF$_2\alpha$ in vivo. These data suggest that the decrease in adenylate cyclase activity during luteolysis was due to an alteration between the $N_S$ and $C$. Alterations in the physical properties of the plasma membrane could affect other enzymes as well. Kim and Yeoun (1983) reported that PGF$_2\alpha$ induced a decrease in $Na^+ - K^+ -$ATPase activity of rat luteal membranes within one hour of incubation. This effect of PGF$_2\alpha$ was due either to a reduction in the number of enzyme molecules, or to masking the active site of the enzyme, the latter of which could occur upon disruption of plasma membrane structure.

Prolonged exposure to PGF$_2\alpha$ results in decreases in LH receptors (Diekman et al., 1978a,b), decreases in LH-receptor aggregation (Luborsky et al., 1984), the appearance of gel phase lipid that induces decreased membrane fluidity (Buhr et al., 1979; Carlson et al., 1982, 1984; Goodsaid-Zalduondo et al., 1982), increases in lysosomal
enzyme activity (McClellan et al., 1977), and decreased steroidogenic enzyme activity (Behrman et al., 1971). The combination of these events, plus more that remain to be characterized, would culminate in complete luteolysis.

It is tempting to speculate a "Yin/Yang" mechanism for the effects of PGE$_2$ in altering PGF$_2\alpha$ action. The importance of PGE$_2$ as a possible signal from the conceptus during early pregnancy has been documented (Niswender et al., 1985b). However, the two hormones appear to have different mechanisms of action. Specific receptors for PGE$_2$ also exist in bovine (Rao, 1976) and ovine (Fitz et al., 1982) luteal membranes, and PGE$_2$ has been shown to stimulate adenylate cyclase activity in rabbit (Abramowitz and Birnbaumer, 1979) and ovine (Fletcher and Niswender, 1982) luteal cells. Prostaglandin E$_2$ appears to influence a separate pool of adenylate cyclase in ovine luteal cells because pretreatment with PGF$_2\alpha$ does not affect the ability of PGE$_2$ to stimulate this enzyme. Thus, PGE$_2$ could act at the level of the luteal cell to maintain progesterone synthesis in the presence of PGF$_2\alpha$ that is still observed in the uterine venous blood of ewes during early pregnancy (McCracken et al., 1984). The cellular mechanism(s) whereby PGE$_2$, in the presence of PGF$_2\alpha$, maintains luteal function have not been studied.

The possible interactions among LH, PGF$_2\alpha$ and PGE$_2$ on luteal plasma membrane functions become more complex if one considers the proposed contributions of small and
large cells to luteal progesterone synthesis (Silvia et al., 1984a, Fitz et al., 1984b; Niswender et al., 1985b). The collective hypothesis proposed by Niswender and his researchers states that the large cells respond to prosta-
glandins, and appear to influence the function of the small cells. Thus, during luteolysis, PGF$_2$α would inhibit progesterone synthesis in the large cells, that contribute to the majority of steroidogenesis, and could also cause the release of some "toxin" or factor that would inhibit small cell function. Similarly, during early pregnancy, PGE$_2$, acting on the large cells would prevent PGF$_2$α-induced decreases in steroidogenesis. It must be remembered, however, that LH is absolutely required for the mainten-
ance of pregnancy in ewes, thus LH-stimulated events in the small cell must also be important in the overall regulation of luteal function. Niswender et al. (1985b) propose that LH may be necessary for the transformation of small cells to large cells, as well as stem cells to small cells. There may be additional mechanisms whereby the two cell types communicate that would not be evident from experimentation performed with large and small cells in separate cultures. "Septate-like" junctions have been observed between adjacent ovine luteal cells by electron microscopy (McClellan et al., 1975), but their function has not been characterized. Thus, further research is necessary before the specific interactions that occur between large and small cells can be delineated, and may
help to explain how events initiated at their respective plasma membranes control luteal function in ewes.
STATEMENT OF THE PROBLEM

During the past decade, some outstanding increases in animal production have been realized through the use of artificial insemination, estrous synchronization, nonsurgical embryo transfer, hormonal induction of ovulation out of season, and increasing ovulation rate with androgen antisera. The ultimate goals of these reproductive manipulations are to increase the genetic superiority and the number of offspring born per female. Yet, approximately 30% of the fertilized ova in domestic animals are lost during the first few weeks of gestation (Edey, 1979), with less than 5% of embryonic mortality represented by chromosomal abnormalities (Boyd, 1965) and fertilization failure (Edey, 1979). The physiological basis for early embryonic loss is complex and is currently the subject of intense research. Embryo survival may be compromised by insufficient luteal development resulting in a maternal uterine environment that is abnormal or unable to support implantation, inadequate production by the embryo of the appropriate substances necessary for maternal recognition of pregnancy, the inability of the dam to recognize these embryonic signals, asynchrony between the developmental age of the embryo and the appropriate uterine milieu established by the precise duration of exposure to progesterone, inappropriate distribution of embryos within the uterus, and inadequate responses of the embryonic or
maternal immune systems resulting in rejection of the fetal allograft. None of these causes for decreased embryo survival may be mutually exclusive, and they indicate the difficulty in arriving at simple, practical methods for improving reproductive efficiency.

Reproductive efficiency in ewes is largely related to the number of lambs weaned/ewe/year; thus, increasing the number of lambs born/ewe as well as the number of lambings/year could favorably influence the sheep industry. It has been estimated that by improving reproductive techniques alone, a 13% gain in the efficiency of the sheep industry over a ten-year period would result and manifest itself in an additional 5 lbs. meat/ewe, $0.91 return/ewe and $8.5 million/year (Gerrits et al., 1979).

But paramount to realizing these economic advantages is the need to understand why prenatal losses occur despite natural selection for efficient reproduction. The basic mechanisms underlying the ability of the ovine embryo to thwart luteolysis, thereby ensuring its survival, have only been studied within the last five years. In this short period, exciting advances have been made in identifying the embryonic signals, namely oTP-1 and PGE_2, as well as in the mechanisms of action of LH and PGF_2α at the level of the ovine luteal cell. However, little if any information existed concerning the physical properties of the cellular organelle that provides the locus for hormonal regulation of progesterone production,
the luteal cell plasma membrane. The desire to initiate new knowledge regarding the characteristics of luteal cell plasma membranes during luteolysis and early pregnancy in the ewe provided the impetus for the following studies.
CHARACTERIZATION OF PLASMA MEMBRANE LIPIDS
AND LUTEINIZING HORMONE RECEPTORS OF OVINE CORPORA LUTEA
DURING LUTEOLYSIS AND EARLY PREGNANCY

Introduction

The presence of a viable conceptus in the uterus by days 12-13 post-mating is necessary for the prevention of corpus luteum (CL) regression and the establishment of a successful pregnancy in the ewe (Moor and Rowson, 1966a,b). Regression of the ovine CL during the estrous cycle is believed to be caused by uterine endometrial secretion of prostaglandin F$_2$α (PGF$_2$α; McCracken et al., 1972), which is transported in a local manner to the ovary (Ginther et al., 1973). The conceptus appears to interfere with the luteolytic activity of PGF$_2$α because the uterine secretion of PGF$_2$α continues during early pregnancy (for review, see Inskeep and Murdoch, 1980). Recent evidence suggests the ovine conceptus produces a unique protein, ovine trophoblast protein (oTP-1), and possibly additional proteins, synthesized transiently between days 13 and 21 of pregnancy, which can prolong luteal maintenance when infused into the uterine lumen of cyclic ewes (Godkin et al., 1982; Godkin et al., 1984a,b). Prostaglandin E$_2$ (PGE$_2$) of uterine and/or conceptus origin has also been implicated as an important factor involved in luteal maintenance during early pregnancy in the ewe (for
review, see Silvia et al., 1984a).

In addition to the above embryonic and uterine factors, luteinizing hormone is absolutely required for the normal synthesis and secretion of progesterone in the ewe (Short, 1964; Hansel et al., 1973; Nalbandov, 1973; Niswender et al., 1980). Hypophysectomy performed on days 3 and 10 of gestation caused complete luteal regression and abortion by day 20 (Denamur, 1974). Continuous infusions of crude LH, but not other pituitary hormones, maintained progesterone secretion and a viable embryo in hypophysectomized pregnant ewes (Kaltenbach et al., 1968b). Although it remains controversial at present whether the ovine conceptus proteins and/or PGE2 can and must act directly on the luteal cell (Mapletoft et al., 1976; Godkin et al., 1978; Ellinwood et al., 1979a; Niswender et al., 1982; Godkin et al., 1984a), it is necessary that they function in concert with LH to ensure the maintenance of progesterone secretion and thus, pregnancy.

The importance of the plasma membrane in the regulation of luteal cell function centers around the mechanism of action of LH. It is currently believed that subsequent to the interaction of LH with plasma membrane receptors, the activation of adenylate cyclase leads to increased intracellular levels of adenosine 3':5'-monophosphate (cAMP) and increased protein kinase activity which influences progesterone synthesis by a variety of mechanisms (Marsh, 1976; Niswender et al., 1980, 1982). A role for
the phospholipid components of the plasma membrane in providing a stable environment for ligand-receptor interactions of the glycoprotein hormones hCG (Ahzar and Menon, 1976; Ahzar et al., 1976), FSH (Abou-Issa et al., 1976; O’Neill and Reicher, 1984) and TSH (Aloj et al., 1979) as well as for the activation of adenylate cyclase by many hormones (Levey and Lehotay, 1976; Schier et al., 1976) has been suggested.

According to the collision-coupling hypothesis proposed by Levitski (1978), the plasma membrane lipid milieu provides a matrix through which the hormone receptor complex, presumably located in the outer bilayer, could interact with the guanine nucleotide regulatory protein of the adenylate cyclase enzyme leading to activation of the catalytic subunit, both presumably exposed at the inner bilayer. Thus, modification of membrane fluidity could alter this interaction as shown for the β-adrenergic receptor activation of adenylate cyclase in turkey erythrocytes (Rimon et al., 1978; Hanski et al., 1979; Briggs and Lefkowitz, 1980). Studies using immuno-fluorescent (Amsterdam et al., 1979) and fluorescent photobleaching recovery techniques (Niswender et al., 1985a) provide evidence that the LH-receptor complex moves laterally in the luteal cell plasma membrane. Recent observations using rat (Buhr et al., 1979; Carlson et al., 1981) and bovine (Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982) luteal microsomal membranes as
well as rat luteal plasma membranes (Carlson et al., 1984) revealed that most of the membrane lipid was in a liquid-crystalline, or more fluid state when the CL were secreting progesterone. However, during luteal regression when progesterone secretion is decreased, a portion of the membrane lipid transformed to the gel state with a concomitant decrease in membrane fluidity.

Alterations in membrane fluidity leading to changes in cell function can be influenced in part by the lipid composition of the membrane. Increased membrane fluidity has been observed in many cell types when the phosphatidylcholine:sphingomyelin ratio and degree of unsaturation of the phospholipid fatty acids are increased, as well as when the fatty acid chain length and cholesterol content are decreased (Thompson, 1980; Schachter et al., 1983). Therefore, this study was conducted to determine the plasma membrane lipid composition of ovine corpora lutea during luteolysis and early pregnancy. It was of interest to examine whether the presence of the conceptus was accompanied by altered plasma composition which might facilitate the luteotropic action of LH in maintaining a functional CL. Concentrations of unoccupied LH receptors in the plasma membranes were also quantified as an additional indicator of luteal cell function during these times.
Materials and Methods

Experiment I. Isolation and Analysis of Ovine Corpora Lutea Plasma Membrane Lipids

Animals

Forty mature crossbred ewes were checked twice daily for estrus (Day 0) with a vasectomized ram and were shown to exhibit two consecutive estrous cycles of normal length (15.5±0.1 days). Ewes were assigned randomly to be necropsied on days 13 (D13-NP, n=9) or 15 (D15-NP, n=14) of the estrous cycle or to be mated on the day of estrus and necropsied on days 13 (D13-P, n=9) or 15 (D15-P, n=8) of pregnancy. At necropsy, uteri from mated ewes were flushed with 0.15 M NaCl to verify the presence of a normal elongated blastocyst. Corpora lutea (CL) were quickly dissected from the ovaries, decapsulated, placed in 2.0 ml 0.15 M NaCl (4 C), snapfrozen in a dry ice-methanol bath and stored at -70 C. In order to obtain adequate yields of plasma membranes for lipid analyses, CL collected from each group were allocated randomly to form three separate pools/groups of approximately 3 g luteal tissue/pool. Corpora lutea from ewes that had more than one ovulation were included in the same pool.
Plasma Membrane Isolation

The following procedures are modifications of those utilized by Gospodarowicz (1973a) and Bramley and Ryan (1978) for obtaining bovine luteal and rat ovarian plasma membranes, respectively. All buffers were maintained and procedures performed at 4 °C. While still frozen, CL from each pool (approximately 3 g) were sliced using a Stadie-Riggs blade, placed in a size B Thomas tissue grinder with a size B pestle and homogenized in 20 ml 0.25 M sucrose in 25 mM Tris-HCl (pH 7.4 at 4 °C) - 1 mM CaCl₂ (STC buffer) utilizing eight complete strokes. The homogenizer was rinsed with an additional 10 ml of STC buffer and the total homogenate was filtered through two layers of cheesecloth to remove connective tissue debris prior to centrifugation.

All supernatants and pellets (after gentle resuspension in STC buffer using a Dounce homogenizer with a loosely fitting pestle, size A) obtained at each differential rate centrifugation were recentrifuged at the same speed and time to ensure maximal sedimentation of each subcellular organelle. The supernatants and resuspended pellets obtained from identical centrifugations were combined, the total volumes noted, and 100 μl aliquots removed and stored at -70 °C for protein determination by the method of Lowry (1951). The remaining volume was subjected to the next centrifugation as indicated below.

Each luteal homogenate (30 ml/pool) was centrifuged
at 1000 x g for 10 min, the resultant pellets being referred to as the nuclear fraction. The 1000 x g supernatants were centrifuged at 20,000 x g for 20 min. The pellets obtained following this centrifugation were resuspended in 11 ml of STC buffer and layered over a discontinuous density gradient in a nitrocellulose tube. The gradients consisted of (from bottom to top) 5 ml 50%, 8 ml 46%, 8 ml 40% and 5 ml 30% sucrose (w/w) in 25 mM Tris-HCl (pH 7.4 at 4 C), adjusted to their exact concentration at 25 C using a Bausch and Lomb refractometer. Centrifugation of the gradients for 75 min at 65,000 x g in a Beckman (Palo Alto, CA) SW 27 swinging bucket rotor yielded distinct layers at the interfaces of the different sucrose concentrations. The layers were collected carefully from below, using a glass pipette with a bent tip, starting with the top layer so as not to disrupt the material at the underlying interfaces. The interface overlaying the 30% sucrose layer is called F1; the interface between the 30% and 36% layers is called F2; the interface between the 36% and 40% layers is called F3; and the bottom layer between the 40% and 50% sucrose is called F4. Each interface was then diluted 1:2 with 25 mM Tris-HCl (pH 7.4 at 4 C) - 1 mM CaCl2, centrifuged at 100,000 x g for 60 min and the resultant pellets extracted for lipid analysis (see below). The 20,000 x g supernatants were centrifuged for 60 min at 100,000 x g to sediment the microsomes with the resulting supernatant repre-
senting the cytosol fraction.

**Enzyme Assays**

In order to determine which fraction obtained by centrifugation was enriched in plasma membranes and the extent to which it was contaminated by other subcellular organelles, marker enzyme assays were performed. Eight corpora lutea collected from five additional D13-NP ewes were separated into three pools of approximately 1.5 g/pool. Each pool of luteal tissue was subjected to the homogenization and centrifugation procedures as described above, and the resulting pellets were gently resuspended in a known volume of STC buffer. These pellets and the 100,000 x g cytosol fraction were then snap-frozen and stored at -70 C. All enzyme assays were conducted two days after collection of the fractions.

The optimal temperature and substrate concentrations for the various marker enzymes were determined prior to their assay in subcellular organelles using whole homogenates of corpora lutea from D13-NP or D13-P ewes. Characterization of each enzyme activity was conducted in triplicate using three separate homogenates, including appropriate blanks and controls, and activity was shown to be linear with respect to time and protein concentration. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA, fraction V, Sigma Chemical Co., St. Louis, MO) as the standard.
5‘-Nucleotidase (EC 3.1.3.5). The activity of 5‘-nucleotidase, a plasma membrane marker enzyme, was assayed according to the method of Michell and Hawthorne (1965). The 2.0 ml assay system consisted of 1.9 ml 5 mM adenosine-5‘-phosphate (Sigma) - 100 mM KCl - 10 mM sodium potassium tartrate - 10 mM MgCl₂·6H₂O in 50 mM Tris-HCl, pH 7.4 and was initiated with 0.1 ml of tissue fraction. After incubation at 37°C for 15 min, 1.0 ml ice-cold 25% TCA (Sigma) was added to stop the reaction and the tubes were centrifuged at 1500 x g for 10 min. Aliquots (2.0 ml) of the supernatant were analyzed for inorganic phosphate (Pi) by the method of Bartlett (1959). The 5‘-nucleotidase activity at 37°C was linear over 25 min at 0.1, 0.25, 0.5 and 1.0 mg CL homogenate protein and is expressed as μmole Pi released·min⁻¹·mg protein⁻¹.

NADPH-cytochrome c reductase (EC 1.6.2.4). NADPH-cytochrome c reductase activity was used as a marker for endoplasmic reticulum membranes (Sottocasa et al., 1967) although some activity is also present in Golgi fractions. Activity was monitored according to LaDu (1971) in a 3.0 ml system by adding in the following order: 2.0 ml 3 mM KCN - 60 mM nicotinamide - 70 μM freshly prepared NADPH (Sigma) - 1 mM EDTA in .05 M potassium phosphate, pH 7.6; 0.5 ml 300 μM cytochrome c (Sigma, Type III) freshly prepared in distilled water; and 0.5 ml of tissue fraction. The rate of reduction of cytochrome c was
followed spectrophotometrically by observing the increase in optical density at 550 nm. Activity was calculated using $E = 29.5 \text{ mM}^{-1}\text{cm}^{-1}$ for reduced cytochrome c at 550 nm and expressed as nmoles cytochrome c reduced·min$^{-1}$·mg protein$^{-1}$. The reaction was linear for up to 7 min at 25 C with 0.25, 0.5, 0.75 and 1.0 mg CL homogenate protein. Attempts to measure NADPH-cytochrome c reductase activity according to Bramley and Ryan (1978) at a pH of 9.5, with the inclusion of 0.1% Triton X-100 in order to abolish NADP-cytochrome c reductase (EC 1.6.99.3) and cytochrome oxidase (EC 1.9.3.1) activities, resulted in reaction rates that were linear for only 30 sec when CL homogenates, 20,000 x g pellets or microsomal pellets were used.

**Succinate dehydrogenase (EC 1.3.99.1).** The activity of succinate dehydrogenase was used as an indicator of the presence of mitochondrial membranes. Activity was assayed using a modification of the method of King (1967). The 3.0 ml reaction mixture consisted of 2.0 ml 100 mM Tris-HCl, pH 7.4, 0.2 ml 50 mM KCN, 0.2 ml 2.5 mM phenazine methosulfate (Sigma), 0.2 ml 250 mM sodium succinate, 0.2 ml of tissue fraction and was initiated by the addition of 0.2 ml 4 mM 2,6-dichlorophenolindophenol (DCPIP, Sigma). The rate of reduction of DCPIP at 37 C was followed by observing the decrease in optical density at 600 nm. The reaction was linear for up to 7 min using
0.5, 0.75 and 1.0 mg CL homogenate protein. An E = 18.3 mM⁻¹ cm⁻¹ of reduced DCPIP at 600 nm was used to calculate specific activity which is expressed as nmoles DCPIP reduced·min⁻¹·mg protein⁻¹. Reduction of DCPIP was not observed when sodium succinate was replaced by sodium malonate, a specific inhibitor of succinate dehydrogenase. This indicated that DCPIP was being reduced by succinate dehydrogenase and not by other enzymes in the homogenate capable of reducing artificial substrates (Evans, 1978).

Plasma Membrane Lipid Analysis

Lipid Extraction. Lipids were extracted from the washed pellets obtained from the combined F1 and F2 fractions by the method of Bligh and Dyer (1959). The pellets were homogenized in 3.75 ml chloroform-methanol-deionized distilled water (CHCl₃-CH₃OH-DDH₂O; 2:1:0.8, vol/vol) in a Dounce homogenizer and stored under nitrogen at 4°C overnight. The next day, 2.5 ml CHCl₃-DDH₂O (1:1, vol/vol) was added and the samples (under nitrogen) were centrifuged for 30 min at 800 x g and 25°C in a swinging bucket rotor. The lower CHCl₃ layer (lipid extract) was carefully removed and quantitatively transferred to an acetone-wiped, preweighed screw-cap tube and dried under nitrogen. The lipid extracts were desiccated over fresh KOH pellets under vacuum, exposed to nitrogen and weighed. The lipids were dissolved in benzene (1 mg lipid/25 µl) and stored under nitrogen at -20°C.
Phospholipids. Plasma membrane phospholipids were separated using two-dimensional thin layer chromatography (TLC) on 0.5 mm silica gel plates containing 5% magnesium acetate (Supelco, Inc., Bellefonte, PA). Plates were prewashed in CHCl₃:CH₃OH:DDH₂O (65:25:4, vol/vol) and activated at 110°C for 60 min prior to use. Approximately 800 µg lipid extract was transferred quantitatively to the plate under nitrogen. Duplicate plates for each CL pool were prepared. Plates were developed in paper-lined tanks in the first dimension in CHCl₃:CH₃OH:28% ammonium hydroxide (65:35:5, vol/vol/vol), dried for 30 min under nitrogen, rotated 90° and developed in the second dimension in CHCl₃:acetone:CH₃OH:glacial acetic acid:DDH₂O (30:40:10:10:5, vol/vol; Rouser et al., 1970). Plates were air dried and phospholipids visualized by brief exposure to iodine vapor. Phospholipid areas were located by comparison to migration of pure standard phospholipids (Sigma). Each phospholipid spot and a blank area were scraped from the plates into separate tubes, digested for 1 h at 180°C with 1.0 ml 72% perchloric acid, cooled and quantified by measurement of Pi using a procedure reported by Dittmer and Wells (1969) as follows. To each sample was added 4.5 ml ammonium molybdate (0.4% in DDH₂O, wt/vol) and 0.2 ml aminonaphthol-4-sulfonic acid reagent (ANSA; Sigma; 0.2% ANSA, 12% sodium bisulfate and 2.4% sodium sulfite in DDH₂O, wt/vol). Samples were vortexed, heated in a waterbath at 100°C for 10 min, cooled, centri-
fuged to sediment the silica gel and absorbance read at 830 nm. A standard curve ranging from 0 to 10 μg Pi as monobasic potassium phosphate was used to calculate the amount of Pi released from the phospholipids. In addition, a portion of the lipid extract was quantified for total phospholipid Pi. Sample phospholipid values were corrected by subtracting Pi present in the blank spots and by dividing by the recovery of total phospholipid Pi, which ranged from 50 to 200 ng and 65 to 96%, respectively. Micrograms of Pi present in the sample were multiplied by 25 (mol.wt. phospholipid/mol.wt. Pi = 775/31) to obtain μg phospholipid. Values are expressed as μg phospholipid/mg membrane protein.

**Fatty acids.** A 1 mg (25 μl) aliquot of lipid extract from each CL pool was subjected to phospholipid analysis in duplicate as described above. After development in the second dimension, plates were dried under nitrogen for 1 h. Phospholipid spots were visualized under UV light after plates were sprayed with 2,7-dichlorofluorescein (Sigma; 0.2% in 90% ethanol, wt/vol). Each spot was scraped from the plate and subjected to transesterification by heating in 4% sulfuric acid in CH₃OH (vol/vol) in sealed tubes for 90 min at 85-90 C. One ml DDH₂O was added to obtain two phases. The methyl esters formed were extracted three times with 2.0 ml hexane, the top layers combined and washed with 5% sodium bicarbonate (wt/vol).
After drying the extract over anhydrous sodium sulphate, it was then filtered through glass wool contained in a disposable pasteur pipette, the pipette rinsed three times with hexane, and the combination filtrate-hexane dried under nitrogen immediately prior to gas liquid chromatography (GLC). The extract was resuspended in 1.0 ml hexane, transferred to a small vial, dried under nitrogen, and 5 to 10 μl of iso-octane were added.

Fatty acid methyl ester (FAME) profiles of each major phospholipid were analyzed with a Varian (Palo Alto, CA) Aerograph series 1200 gas chromatograph. Samples (1-2 μl) were injected onto a 183-cm column packed with GP 10% SP-2330 (cyanosilicone) on 100/120 mesh chromosorb W AW (Supelco) at 190 °C. Identification of peaks was made by comparison of relative retention times with those of polyunsaturated FAME standards (PUFA Nos. 1 and 2, Supelco; NHI-D, Nu Chek Prep, Inc., Elysian, MN) or by determination of equivalent chain length (Hofstetter et al., 1965). Peak areas were recorded and calculated on a Hewlett-Packard 3380A computing integrator (Palo Alto, CA).

Free cholesterol. Approximately 0.4 mg of CL plasma membrane lipid extract was analyzed in duplicate for free cholesterol by the procedure outlined by Kates (1972). Due to lack of sufficient lipid, only free, or unesterified, cholesterol was measured since most (> 80%) of the cholesterol in plasma membranes is present in this form (Evans,
The extract was placed into a 15 ml conical centrifuge tube and dried under nitrogen. One ml of acetone:95% ethanol (1:1, vol/vol) was added, followed by 1.0 ml of 1% digitonin (Sigma; wt/vol dissolved in 95% ethanol:DDH2O, 1:1, vol/vol). This mixture was incubated for 10 min at 25 C and then centrifuged at 2000 x g for 5 min. The supernatant was discarded, the precipitate was allowed to drain for 5 min and dried under nitrogen. The digitonide precipitate was dissolved in 3.0 ml glacial acetic acid and transferred quantitatively to another tube; the original tube was rinsed with an additional 3.0 ml acid and added to the remainder. Four ml of a dilute ferric chloride color reagent (2.5% FeCl₃·6H₂O in concentrated orthophosphoric acid [wt/vol] diluted 8% in concentrated sulfuric acid [vol/vol]) was added to the acetic acid, carefully vortexed and allowed to cool. Absorbance was read at 550 nm, and mg free cholesterol in the sample was calculated from a standard curve ranging from 25 to 300 µg cholesterol (Sigma) dissolved in glacial acetic acid.
Experiment II. Analysis of Corpora Lutea Plasma Membrane LH Receptors

**Animals and preparation of luteal plasma membranes**

Twenty-four mature crossbred ewes were assigned randomly (6/group) to be ovariectomized on days 13 (D13-NP) or 15 (D15-NP) of the estrous cycle or mated on the day of estrus and necropsied on days 13 (D13-P) or 15 (D15-P) of pregnancy. Uteri of pregnant ewes were flushed to verify the presence of a viable embryo. Corpora lutea were dissected, decapsulated, placed in 0.15 M NaCl, snap-frozen and stored at -70 C for less than 2 months.

On the day of assay, plasma membrane fractions (F1 and F2) from corpora lutea of individual animals were prepared as described in Experiment I. The protein content of the plasma membrane fractions was determined using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with BSA (Sigma) as the standard.

**Preparation of radioiodinated hCG**

Highly purified hCG (CR-121; 13,450 IU/mg; supplied by the National Hormone and Pituitary Agency, NIADDK) was radioiodinated according to the lactoperoxidase method (Thorell and Johansson, 1971) as described by Catt et al. (1976). Separation of labeled hormone from free iodine was performed by filtration on a Sephadex G-75 column (0.7 x 24 cm) previously equilibrated and eluted with
0.05 M Tris-HCl (pH 7.4) - 0.1% BSA. Following the collection of thirty 0.5 ml fractions, two peaks of radioactivity were obtained, with the $^{125}$I-iodo-hCG eluting in the first peak (fractions 10-13) which also represented the void volume. Unreacted iodine eluted in the second peak. Further purification of the iodinated hormone from BSA and/or aggregated components was conducted by elution through a column of Sephadex G-100 (0.9 x 50 cm) equilibrated with Tris-HCl-BSA buffer. Two peaks of radioactivity were again observed after the collection of fifty 1.0 ml fractions, the first peak (fractions 15-18) representing $^{125}$I-iodo-hCG and the second peak (fractions 35-40) consisting of free iodine. Labeled hCG obtained from the second purification was stored at 4 C and used within 10 days of iodination. It has been shown that the biological activity of the native hCG preparation in stimulating progesterone and testosterone production by dispersed ovine luteal cells (Diekman et al., 1978a) and rat testicular homogenates (Catt et al., 1976), respectively, is retained after iodination using lactoperoxidase.

**Determination of maximum binding and specific activity**

The proportion of radioactivity which represents biologically active hormone not damaged by the iodination procedure was determined by incubating a constant amount of $^{125}$I-iodo-hCG (25,000 cpm/tube) with increasing
amounts of plasma membrane protein in the presence or absence of 250 ng unlabeled hCG (Pregnyl; 1665 IU/mg; Organon Pharmaceutical, West Orange, NJ) at 25 C for 16 h. The amount of specifically bound $^{125}$I-iodo-hCG was calculated by subtracting nonspecific binding (membranes incubated with labeled hCG and Pregnyl) from total binding (membranes incubated with labeled hCG alone) after separation of bound from free $^{125}$I-iodo-hCG as described below. The specific binding of labeled hormone expressed as a percentage of the total cpm added and plotted as a function of increasing membrane protein is shown in Figure 1. The active hormone fraction, which represented maximum binding, varied with each iodination and ranged from 42 to 55% in the presence of 100 μg membrane protein. Nonspecific binding remained constant at 5% of the total radioactivity added at all membrane protein concentrations. In addition, specific binding of labeled hCG is shown to be linear up to 100 μg of membrane protein, and aliquots of 50 μg were used in the remainder of the studies. The radioactivity specifically bound to luteal membranes is believed to represent only intact hCG; therefore, the specific activity curves and total radioactivity added in the Scatchard analyses were corrected for maximum binding.

The specific activity of each preparation of radioiodinated hormone was determined by self-displacement analysis (Ketelslegers et al., 1975) utilizing plasma membranes prepared from corpora lutea of four additional
Figure 1. Maximum of $[^{125}I]$iodo-hCG (2.5x10^3 cpm/tube) bound during incubation with increasing concentrations of luteal plasma membrane protein. Each point represents the mean ± SE of two separate pools of CL (1 g/pool) from D13-NP and D13-P ewes from which plasma membranes were obtained.
ewes necropised on D13-P or D15-P (Figure 2). The relative ability of increasing concentrations of unlabeled hCG (0.1-100 ng/tube) to inhibit the binding of a constant quantity (25,000 cpm) of the \[^{125}\text{I}i\text{odo-hCG}\] (Curve A) to plasma membranes was compared with the total binding of labeled hCG obtained by incubating membranes with increasing concentrations of \[^{125}\text{I}i\text{odo-hCG}\] (1-50 x 10^{-4} cpm/tube, Curve B). Two preparations of labeled hCG were used in this experiment. The specific activity of each preparation, 48.5 and 42.9 μCi/μg, was calculated using a weight of 46,000 daltons for hCG (Morgan and Canfield, 1971), 66% counting efficiency and corrected for maximum binding.

**Measurement of hCG binding**

The binding of labeled hCG to luteal plasma membranes of individual ewes was determined by the method of Diekman et al. (1978a) with modifications. Saturation analysis was performed in 12 x 75 mm conical polystyrene tubes (Sarstedt, Princeton, NJ) by adding 50 μl membrane suspension (1 mg membrane protein/ml 25 mM Tris-HCl, pH 7.4, 1 mM CaCl\(_2\)) in triplicate from each of three ewes in each group to increasing concentrations of \[^{125}\text{I}i\text{odo-hCG}\] (6x10^{-3} - 5x10^{-5} cpm/tube) or with labeled hCG plus a 1000-fold excess of Pregnyl in a final incubation volume of 0.5 ml Tris-CaCl\(_2\) buffer containing 0.5% BSA. Following incubation for 16 h at 25 C, bound and free
Figure 2. Determination of specific activity of radioiodinated hCG by self-displacement analysis. Curve A represents the percentage specific binding of $2.5 \times 10^3$ cpm labeled hCG/tube in the presence of increasing amounts of unlabeled hCG. Curve B depicts total binding of increasing amounts of labeled hCG. Each tube contained 50 µg luteal plasma membrane protein. Specific activity is calculated by dividing the cpm obtained at a B/T ratio of 50% from Curve B by the quantity of unlabeled hCG that displaced 50% of the labeled hCG in Curve A. In this case, specific activity = $3.1 \times 10^4$ cpm/2.4 ng which converts to 48.5 µCi/µg when corrected for maximum binding. Each point represents the mean of plasma membranes prepared from two separate pools of CL (1 g/pool) obtained from a total of four D13-P or D15-P ewes.
hormone were separated by precipitation of the bound fraction with polyethylene glycol (PEG; MW 6000-7500, J. T. Baker Chemicals, NJ) as described by Bramley and Ryan (1978). Briefly, 0.5 ml of cold 0.5% bovine-γ-globulin (wt/vol in Tris-CaCl₂; Sigma) was added to each tube and mixed. An equal volume of 20% PEG was then added, tubes were vortexed and centrifuged at 1500 x g for 10 min at 4°C. After careful aspiration of the supernatants, the pellets were resuspended by vortexing in 1.0 ml 25 mM Tris-HCl, pH 7.4, and 1.0 ml PEG was added again. The precipitates were collected as described above and the radioactivity present in the pellet was counted.

Characteristics of the specific binding of [¹²⁵I]iodo-hCG to corpora lutea plasma membranes of D13-NP ewes are presented in Figure 3. These data indicate that a concentration of 10.5 fmol/tube (1.2 x 10⁻⁵ cpm) saturates the LH receptor in 50 µg plasma membrane protein. Specific binding of labeled hCG to luteal plasma membranes of D15-NP, D13-P and D15-P ewes was also found to be saturated at this concentration (data not shown). Non-specific binding of hCG represented 25±2, 19±5, 10±5 and 13±3% of the total binding of D13-NP, D15-NP, D13-P and D15-P luteal plasma membranes, respectively.

The specific binding of hCG to luteal plasma membranes, 20,000 x g pellet, F3, F4 and microsomal fractions obtained from the remaining three ewes/group was also assessed using 50 µg protein/tube and the single
Figure 3. Saturation analysis of $^{125}\text{I}$ido-hCG binding to ovine corpora lutea plasma membranes. Increasing concentrations of labeled hCG were incubated in triplicate in the absence (total) or presence (nonspecific) of a 1000-fold excess of Pregnyl. The difference between total and nonspecific represents specific binding. Each point is the mean ± SE of luteal plasma membranes obtained from D13-NP ewes (n=3).
Quantification of serum progesterone

Immediately prior to necropsy or ovariectomy, a jugular blood sample was collected from each ewe utilized in both experiments for the quantification of serum progesterone by radioimmunoassay (Koligian and Stormshak, 1977).

Statistics

Data from both experiments were analyzed by 2x2 factorial analysis of variance with day of the estrous cycle or pregnancy (Day) and parity (Stage) as main effects. Preplanned orthogonal contrasts (D13-NP vs. D15-NP, D13-NP vs. D13-P, and D13-P vs. D15-P) were made to determine differences among means when main effects were significant. Data from the saturation analyses of hCG binding to luteal membranes were analyzed by least-squares linear regression and expressed according to the methodology of Scatchard (1949).
Weights of corpora lutea collected during both experiments from D13-NP, D13-P and D15-P ewes were similar (526.4±19.3, 572.4±18.9, and 525.0±26.8 mg, respectively; mean±SE) and greater than (P < 0.01) corpora lutea from D15-NP ewes (423.8±24.3 mg). Consequently, the serum progesterone level of D15-NP ewes was less (0.5±0.09 ng/ml; P < 0.01) than that of D13-NP, D13-P and D15-P ewes (1.9±0.15, 1.7±0.14 and 2.2±0.20 ng/ml, respectively). These data indicate that functional luteolysis was occurring in D15-NP ewes and that the presence of a blastocyst on D13 and D15-P maintained progesterone secretion.

The distribution of protein and marker enzyme activities of ovine corpora lutea subcellular fractions obtained after differential and sucrose gradient centrifugation is shown in Table 5. Total recovery of protein, 5'-nucleotidase, NADPH cytochrome c reductase and succinate dehydrogenase activities were 98±7, 56±10, 57±6 and 39±9%, respectively. Considerable enzyme activity was lost from the 20,000 x g pellets during subsequent fractionation in the sucrose gradients. However, the protein content of fractions F1 through F4, when combined, represented 86% of the protein present in the 20,000 x g pellets prior to
Table 5. Distribution of protein and marker enzyme activities of ovine corpora lutea subcellular fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>5'-Nucleotidase</th>
<th>NADPH cytochrome c reductase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%a</td>
<td>SAb</td>
<td>SAC</td>
</tr>
<tr>
<td>Homogenate</td>
<td>45.3±4.8</td>
<td>100</td>
<td>181.5±31.6</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>8.0±2.0</td>
<td>17.4±3.2</td>
<td>63.8±5.2</td>
<td>15.6±3.4</td>
</tr>
<tr>
<td>F1</td>
<td>1.7±0.2</td>
<td>4.2±0.7</td>
<td>958.2±390.0</td>
<td>21.4±2.5</td>
</tr>
<tr>
<td>F2</td>
<td>1.2±0.1</td>
<td>3.0±0.2</td>
<td>727.2±235.5</td>
<td>9.3±2.0</td>
</tr>
<tr>
<td>F3</td>
<td>1.1±0.1</td>
<td>2.2±0.6</td>
<td>39.3±12.7</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>F4</td>
<td>1.5±0.4</td>
<td>2.7±0.5</td>
<td>61.7±14.2</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Microsomes</td>
<td>2.0±0.4</td>
<td>4.3±0.4</td>
<td>515.5±90.5</td>
<td>10.8±1.3</td>
</tr>
<tr>
<td>Cytosol</td>
<td>28.9±2.4</td>
<td>64.4±3.9</td>
<td>40.0±8.10</td>
<td>ND</td>
</tr>
</tbody>
</table>

Three separate pools of approximately 1.5 g luteal tissue/pool were homogenized and subjected to differential centrifugation as described in the text. The various membrane fractions were resuspended and assayed for protein and marker enzyme activities (see text). Values represent mean±SE from the three replicates.

a Percentage recovery = total protein or enzyme activity of fraction/total protein or activity of homogenate
b Specific activity expressed as nmoles Pi liberated·min⁻¹·mg protein⁻¹.
c nmoles cytochrome c reduced·min⁻¹·mg protein⁻¹
d nmoles DCPIP reduced·min⁻¹·mg protein⁻¹
e Nondetectable
centrifugation in the gradients. Based on the distribution of 5'-nucleotidase activity, fractions F1 and F2 were designated as the plasma membranes. Contamination of the plasma membrane fraction by smooth endoplasmic reticulum as represented by NADPH cytochrome c reductase activity and by mitochondria as indicated by succinate dehydrogenase activity was low, but evident as 5.9 and 1.1%, respectively, of the total enzyme activity in the homogenate for F1, and 3.9 and 2.8% respectively for F2.

Figure 4 illustrates a five- and four-fold enrichment (with respect to the homogenate) of 5'-nucleotidase activity for F1 and F2, respectively. The microsomal fraction showed the greatest enrichment, five-fold, in NADPH cytochrome c reductase activity. The activity of succinate dehydrogenase exhibited little enrichment in any fraction, which may be due to the low recovery of total enzyme activity. However, the majority of the recoverable activity sedimented in the 1000 x g fraction which is similar to the sedimentation profile of this enzyme reported by Powell et al. (1976) for bovine corpora lutea membranes.

The data in Table 5 and Figure 2 indicate that fractions F1 and F2 appear to yield the greatest specific activity for the surface membrane marker, with the lowest contamination by the endoplasmic reticulum and mitochondria. It is recognized, however, that these intracellular organelles are present in F1 and F2 obtained by the centrifugation scheme employed in this study. In order to
Figure 4. Profiles of the enrichment of marker enzyme activities in ovine corpora lutea subcellular fractions obtained by centrifugation as described in the text. The ordinate represents relative specific activity (specific activity in the fraction/specific activity in the homogenate) of the marker enzymes. The cumulative percentage of homogenate protein recovered in each fraction is plotted on the abscissa. The bars designated as F1 and F2 represent the fractions most enriched in plasma membranes. Bars represent mean ± SE from three separate pools of luteal homogenates.
obtain enough material for the lipid and receptor analyses, F1 and F2 were combined. Approximately 1-2 mg plasma membrane protein/g CL tissue, which represented 3.5% of the total homogenate protein, was routinely isolated in F1 and F2 combined.

The phospholipid composition of ovine corpora lutea plasma membranes from ewes necropsied on D13 or D15 of the estrous cycle or pregnancy is depicted in Figure 5. Two-dimensional TLC revealed the presence of the following predominant phospholipids (and their percentage of the total phospholipid) in luteal plasma membrane lipid extracts obtained from ewes in all groups: PC (48.9 ±0.6%), PE (33.3±0.4%), sphingomyelin (SPH, 9.7±0.3%), phosphatidylserine (PS, 3.5±0.2%) and phosphatidylinositol (PI, 4.0±0.5%). Two additional spots that migrated to the identical area as the reference standard cerebrosides were observed in each lipid extract, but were not further characterized. Phosphatidic acid (PA) and lysophosphatidylcholine were identified in trace amounts. There was no visual evidence of phosphatidylglycerol or diphosphatidylglycerol (cardiolipin) in any lipid extract.

The data in Figure 5 indicate that there are no significant differences in the concentrations of total phospholipids, PC, PE, SPH, PS or PI present in plasma membranes isolated from ovine CL on days 13 or 15 of the estrous cycle or pregnancy. In addition, the percentage of the total phospholipid represented by each phospholipid
Figure 5. Concentrations of the total and major classes of phospholipids present in ovine corpora lutea plasma membranes. Lipids were extracted from plasma membranes and phospholipids analyzed by TLC as described in the text. Each bar represents the mean ± SE of duplicate determinations of plasma membrane lipid extracts obtained from each of three separate pools of CL tissue/group. PC = phosphatidylcholine; PE = phosphatidylethanolamine; SPH = sphingomyelin; PS = phosphatidylserine; PI = phosphatidylinositol.
as indicated above was not significantly different among all groups.

Figures 6 through 9 reveal the fatty acid composition identified after transesterification of PC, PE, PS and PI, respectively. The fatty acid composition of SPH is not presented due to incomplete hydrolysis of the N-acyl bonds between the sphingosine base and fatty acid using the procedure for obtaining FAME employed herein.

As shown in Figure 6, no significant differences in the relative percentages of the two major fatty acids present in PC, palmitic (16:0) and oleic (18:1), were observed. The percentage of icosatetraenoic acid (20:3) was greater ($P < 0.05$) in PC of CL plasma membranes collected from nonpregnant as compared to pregnant ewes. The arachidonic acid (20:4) content of PC was greater ($P < 0.05$) in D15-NP than in the other three groups. A Stage x Day interaction ($P < 0.01$) in the percentage of docosapentaenoic acid (22:5) resulted from a decrease in this fatty acid from D13 to D15 in nonpregnant ewes as compared to its increase in pregnant ewes.

Approximately 80% of the fatty acid composition of PE consists of stearic (18:0), 18:1, 20:4 and 22:5 as depicted in Figure 7. Plasma membrane PE of luteal tissue from nonpregnant ewes had a greater ($P < 0.05$) content of 22:5 than that of pregnant ewes. Significant changes in the content of some minor fatty acids, linoleic (18:2), icosaenoic (20:1), and 20:3, representing less than 5% of
Figure 6. Fatty acid composition of phosphatidylcholine in plasma membranes prepared from corpora lutea of D13-NP, N15-NP, D13-P and D15-P ewes. The ordinate identifies the various fatty acids observed and the abscissa represents the percentage of each fatty acid relative to the total FAME extracted from the phospholipid and analyzed by GLC as described in the text. Each bar represents the mean ± SE of duplicate determinations from the phospholipid isolated from each of three separate pools of CL tissue/group. Means that are significantly different are indicated by different superscripts.
Figure 6.
Figure 7. Fatty acid composition of phosphatidylethanolamine in ovine luteal plasma membranes. Bars represent mean ± SE. See Figure 6 legend for details.
Figure 8. Fatty acid composition of phosphatidylserine in ovine luteal plasma membranes. Bars represent mean ± SE. See Figure 6 legend for details.
Figure 9. Fatty acid composition of phosphatidylinositol in ovine luteal plasma membranes. Bars represent mean ± SE. See Figure 6 legend for details.
the total, were observed.

The fatty acid composition of PS is represented most notably by 18:0, 18:1 and docosatetraenoic acid (22:4); however, there were no significant differences among groups in these components (Figure 8). There was a greater percentage of both 20:1 (P < 0.01) and 22:5 (P < 0.025) in PS of CL plasma membranes obtained from pregnant ewes than from nonpregnant ewes.

The fatty acids comprising the majority of the total present in PI are 18:0, 20:4 and 22:4 as shown in Figure 9. A significant (P < 0.05) interaction in the percentage of 18:0 is due to the decreased content of this fatty acid from D13 to D15 of the estrous cycle as compared to no change from D13 to D15 of pregnancy, but did not change during similar days of the cycle which tended to result in a significant (P < 0.05) interaction. The PI content of 22:4 was greatest (P < 0.01) in D13-NP and D15-NP than in pregnant ewes on both days, and greater (P < 0.05) in D15-NP than D13-NP ewes. The percentage of 18:1 was greater (P < 0.01) in PI from pregnant as compared to that of nonpregnant ewes. Conversely, the PI content of 22:5 was greater (P < 0.01) in D13 and 15-NP than in D13 and 15-P ewes. The percentage of 20:4 increased from D13 to D15 of pregnancy but did not change during similar days of the cycle, which resulted in a significant Stage x Day interaction (P = 0.05).

Figure 10 illustrates the sum of the saturated (S)
Figure 10. The percentage of the total fatty acid composition of PC, PE, PS and PI present as saturated or unsaturated fatty acids. Bars represent mean ± SE from three pools of CL tissue/group.
and unsaturated (U) fatty acid contents of PC, PE, PS and PI. The only significant differences in the S and U as a percentage of the total were observed in PI. A significant ($P < 0.05$) interaction in S fatty acids resulted due to a decrease from D13 to D15 of the estrous cycle, while S fatty acids of PI from pregnant ewes remained unchanged. In addition, U fatty acids of PI from D13 and D15-NP ewes were greater ($P < 0.05$) than U fatty acids from D13 and D15-P ewes. Unsaturated fatty acids tended to represent a greater portion of the total fatty acids than S fatty acids in all four phospholipids analyzed. However, no significant difference in the U:S ratios of PC, PE or PS were observed in any group. The U:S of PI was greater ($P < 0.025$) in D15-NP (2.73±0.18) than in D13-NP, D13-P and D15-P ewes (1.85±0.17, 1.66±0.15, and 1.78±0.14, respectively).

The free cholesterol content in relation to the total phospholipid concentration of luteal plasma membranes did not change significantly among all four groups. The molar free cholesterol:phospholipid was calculated to be 0.22 ±0.04 (n=3), 0.45±0.09 (n=3), 0.46±0.07 (n=2) and 0.36±0.2 (n=2) for membranes from corpora lutea of D13-NP, D15-NP, D13-P and D15-P corpora ewes, respectively. Differences among these means should, however, be viewed with caution because of the small number of pooled smaples available for analysis.
Experiment II

The specific binding of labeled hCG in various subcellular fractions of luteal tissue from three ewes/group was determined. No significant differences due to Day or Stage in $[^{125}\text{I}]$iodo-hCG specifically bound to aliquots of the 20,000 x g pellet, F1 and F2, F3, F4 or microsomal fractions were observed (data not shown). Therefore, specific binding in each fraction was calculated by totaling results obtained from ewes in all four groups. Specific binding of $[^{125}\text{I}]$iodo-hCG expressed as fmol/total membrane protein for the 20,000 x g pellet (119.3±12.7) was 10-fold greater than that present in the microsomal fraction (26.1±6.3). Approximately 46% of the total LH receptors present in the 20,000 x g pellet was recovered in the F1 and F2, F3 and F4 fractions, which is similar to the observation that most of the 5'-nucleotidase activity was recovered in these fractions after sucrose gradient centrifugation. The specific binding of hCG in the plasma membrane fraction (45.5±6.5) was four-fold greater than that in F3 (9.7±2.1) and nondetectable in F4.

Scatchard analysis of the saturation data for $[^{125}\text{I}]$iodo-hCG specific binding are depicted in Figure 11. These data reveal linear plots ($r^2 = 0.91, 0.95, 0.97$ and 0.95 for D13-NP, D15-NP, D13-P and D15-P, respectively) from which equilibrium dissociation constants ($K_d$) were
Figure 11. Scatchard analyses of the specific binding of $^{131}$Iodo-hCG to ovine luteal plasma membranes. B/F represents specifically bound $^{131}$Iodo-hCG divided by unbound $^{131}$Iodo-hCG. Each point represents the mean ± SE of results obtained from saturation analysis of three ewes/group.
calculated. Analysis of variance revealed no difference in the affinity of the unoccupied receptor for LH in any group.

The concentrations of unoccupied luteal plasma membrane LH receptors quantified on D13 and 15 of the estrous cycle or pregnancy are shown in Figure 12. No differences in the specific binding of $^{125}$Iiodo-hCG expressed as fmol/µg protein or fmol calculated from the total amount of plasma membrane protein recovered per CL were observed in any group.

**Discussion**

Although plasma membranes have been successfully isolated from large amounts of bovine CL tissue pooled from various stages of gestation (Gospodarowicz, 1973; Powell et al., 1976; Rao and Mitra, 1982), this is the first report of the characterization of luteal plasma membranes obtained from ewes. An attempt was made in this study to analyze membrane preparations more homogeneous than previously reported from CL collected at distinct stages of the estrous cycle and early pregnancy. The percentage recovery of each marker enzyme activity with respect to that observed in the total homogenate was somewhat less than that reported for bovine (Powell et al., 1976) and rat (Bramley and Ryan, 1978) CL plasma
Figure 12. Specific binding of [¹²⁵I]iodo-hCG to unoccupied LH receptors in plasma membranes of corpora lutea obtained from ewes on day 13 or 15 or the estrous cycle or pregnancy. Each bar represents the mean ± SE obtained from six ewes. Dark bars indicate the concentration of LH receptors/µg plasma membrane protein, whereas hatched bars depict the level of LH receptors present in the total plasma membrane protein recovered from each ewe.
membranes; however, the percentage recovery and specific activity of 5'-nucleotidase observed in ovine plasma membranes in the present study are very similar to those of the rat (Carlson et al., 1984) and cow (Gospodarowicz, 1973a; Rao and Mitra, 1977, 1982). The distribution profiles and low contamination of ovine luteal plasma membranes by endoplasmic reticulum and mitochondria reported herein agree with those observed for bovine CL (Gospodarowicz, 1973a; Powell et al., 1976). Although it may be argued that the total percentage recovery of succinate dehydrogenase activity in this study may have been too low to adequately assess mitochondrial contamination, the complete absence of cardiolipin, a phospholipid characteristically observed in mitochondrial membranes (Rouser and Fleischer, 1967) from the phospholipid components of the ovine luteal plasma membranes in this study lend support to a low contamination by this organelle.

The presence of endoplasmic reticulum of varying extents in luteal plasma membrane preparations is always reported (Powell et al., 1976; Bramley and Ryan, 1978; Carlson et al., 1984) and is most likely due to the difficulty of separating the vesicles formed by both types of membranes upon cellular disruption (Gospodarowicz, 1973a; Powell et al., 1976). The distribution of specific activities of the various membrane marker enzyme assays was not assessed at each of days 13 and 15 of the estrous cycle and pregnancy in the present study due to lack of sufficient
luteal tissue. However, the distribution and specific activities of mitochondrial and endoplasmic reticulum markers were not altered appreciably in functional or regressing rat CL (Bramley and Rayn, 1978; Carlson et al., 1984) whereas 5'-nucleotidase activity changed in bovine luteal microsomal membranes (Carlson et al., 1982) and rat CL plasma membranes (Bramley and Ryan, 1980) with respect to stage of luteinization.

A paucity of information on the lipid compositions of ovarian tissue obtained from domestic animals exists in contrast to the abundant data available for testicular tissue (Coniglio, 1977; Christie, 1978). The compositional data reported for bovine (Holman and Hofstetter, 1965; Scott et al., 1968), porcine (Holman and Hofstetter, 1965; Waterman, 1980a) and ovine (Waterman, 1980b, 1981, 1982) CL are represented as lipids extracted from tissue homogenates which contain all of the subcellular organelle membranes; thus it is difficult to make direct comparisons with the plasma membrane lipid compositions of ovine CL in this study. Nonetheless, the percentages of the total phospholipid represented by PC, PE, SPH, PS and PI reported herein are very similar to the total phospholipid composition of bovine CL (Scott et al., 1968). In addition, the fatty acid profile of the total phospholipid fractions obtained from ruminant CL during the studies listed above revealed the presence of 16:0, 18:0, 18:1, 18:2, 20:4, 22:4 and 22:5 as the major fatty
acids, which is again identical to those observed in the individual phospholipids reported for ovine luteal plasma membranes, with the proportion of unsaturated fatty acids slightly greater than that of saturated fatty acids. The fatty acid compositions of PC, PE, PS and PI present in ovine luteal plasma membranes are consistent with those generally reported for each phospholipid species in animal tissues, particularly in that PE contains more unsaturated fatty acids than does PC from the same tissue and PI contains a high proportion of 20:4 (Christie, 1973).

In the present study, no changes in luteal plasma membrane concentrations of phospholipids were detected that could be associated with luteolysis or maintenance of functional CL during early pregnancy. Similarly, free cholesterol content of luteal plasma membranes did not vary significantly between nonpregnant and pregnant ewes. In support of our data, Waterman (1981, 1982) did not observe a change in total luteal phospholipid content of ewes on D12 and 14 of the estrous cycle or pregnancy; however, total luteal phospholipids increased in nonpregnant ewes between D13 and 16. Free cholesterol content of total lipids of ovine CL increased slightly from D13 to D15 of the estrous cycle, but was different between D14 pregnant and nonpregnant ewes (Waterman, 1982).

Statistically significant differences in the fatty acid composition of luteal membrane phospholipids were detected between nonpregnant and pregnant ewes as well as
among days within reproductive stages. The majority of these differences involved unsaturated fatty acids of chain length greater than 20 carbons which were present in relatively minor quantities. However, it is not known whether the observed changes in these fatty acids are of biological significance in terms of reflecting the functional status of the corpus luteum. An exception may be the changes detected in arachidonic acid (20:4), which gives rise to the synthesis of prostaglandins. According to the data of Waterman (1981, 1982) the fatty acid composition of total luteal phospholipids of ewes did not undergo any major changes except for a slight increase in arachidonic acid from D15 to 16 to the estrous cycle. In the present study, only arachidonic acid levels of PC and PI varied significantly between nonpregnant and pregnant ewes and among days of the cycle and pregnancy. Perhaps of most relevance are the differences evident in this fatty acid and luteal membranes of ewes on D15 of the cycle and pregnancy. On this given day there was a general trend for arachidonic acid levels of PC and PI in membranes of nonpregnant ewes to be inversely related to those present in membranes of pregnant ewes. In this regard, changes in the levels of docosatetraenoic acid (22:4) and docosapentaenoic acid (22:5) in PI of luteal membranes of nonpregnant and pregnant ewes on D15 are noteworthy. Levels of these PI fatty acids, which are metabolites of arachidonate, were significantly greater in
membranes of nonpregnant ewes than in those of pregnant ewes on D15. In addition, in membranes of D15 nonpregnant ewes the levels of 22:4 in PI were also inversely related to the corresponding levels of 20:4 in this phospholipid. Recently, the initial luteolytic action of PGF₂α on rat luteal cells was shown to involve the rapid hydrolysis of, presumably, membrane phosphoinositides by phospholipase C, resulting in increased generation of intracellular inositol phosphates, and, implicitly, 1,2 diacylglycerol (Leung et al., 1986). Inositol 1,4,5-bisphosphate acts as a second messenger for mobilizing intracellular calcium from the endoplasmic reticulum (Berridge and Irvine, 1984). One possible consequence of calcium mobilization in luteal cells is the activation of phospholipase A₂, which catalyzes the release of arachidonate from the 2 position of the glycerol moiety of PC and PI in many cell systems (Van den Bosch, 1980). No direct conclusions from the present study can be made with regard to the significance of the observed changes in plasma membrane arachidonic acid levels of PC and PI. However, it is possible these changes reflect the availability and(or) the consequences of active metabolism of this fatty acid during luteolysis initiated by uterine PGF₂α and the attenuation of PGF₂α effects on the luteal cell during early pregnancy.

Concentration of unoccupied receptors for LH in luteal plasma membranes quantified on D13 and 15 of the
estrous cycle and pregnancy remained constant. This is in agreement with the data of Diekman et al. (1978a) who found that unoccupied and occupied luteal LH receptor levels were similar on D12 and 14 of the estrous cycle and D12, 16 and 20 of pregnancy. These researchers did observe a significant reduction in receptor number of D16 of the estrous cycle, whereas this was not indicated on D15 in this study. Absolute quantities of unoccupied receptors observed herein are less than those reported by Diekman et al. (1978a), which may be explained by the use of a more purified receptor preparation in this study.

Affinity of the receptor for hCG was also unaltered in luteal plasma membranes from ewes in all groups, indicating that the observed changes in membrane composition did not affect the conformation of the LH receptor. These results also suggest that alterations in LH receptor binding are not a part of the mechanism involved with the maternal recognition of pregnancy in the ewe.

Because of the nature of the present study, certain changes in membrane lipid composition confined to specific cell types or specific domains within the plasma membrane of the corpus luteum may have gone undetected. It is possible that changes in plasma membrane lipids during luteolysis and early pregnancy may have been restricted to a particular luteal cell type. Large ovine luteal cells appear to secrete most of the progesterone in an apparently LH- and cAMP-independent manner, contain few LH
receptors and the majority of the receptors for PGF$_{2\alpha}$ and PGE$_1$, while small luteal cells contain the majority of LH receptors and respond to LH with enhanced progesterone secretion (Fitz et al., 1982; Hoyer et al., 1984). If changes in plasma membrane lipids were manifested according to luteal cell type, this would have been masked in the present study where lipid analyses were performed on membrane preparations contributed by both cell types.

Similarly, localized changes in membrane lipids that affect the ability of luteal cells to respond to LH or the luteolysis could also go undetected by analysis of gross membrane composition. Such local perturbations of membrane lipids, while not affecting the concentration or affinity of LH receptors, as demonstrated in this study, could interfere with the lateral diffusion of the LH-receptor complex in the luteal cell membrane (Luborsky et al., 1984; Niswender et al., 1985a), and(or) activation of adenylate cyclase. Hence, the reduced adenylate cyclase activity that has been reported to occur during PGF$_{2\alpha}$-induced regression of ovine corpora lutea (Fletcher and Niswender, 1982) might be due to the ability of PGF$_{2\alpha}$ to restrict microaggregation of the LH receptor complex in the cell membrane (Luborsky et al., 1984). This latter possibility is strengthened by the observation that decreased fluidity of a lipid probe in rat luteal plasma membranes during normal and PGF$_{2\alpha}$-induced luteolysis occurred in the absence of major changes in membrane lipid
composition (Carlson et al., 1984). Adenylate cyclase activity could also be reduced as a result of an altered interaction between the LH-receptor complex and the guanine nucleotide regulatory subunit brought about by changes in the local lipid environment of these integral membrane proteins.

In conclusion, results of this investigation indicate that major changes in the gross lipid composition of ovine luteal cell plasma membranes are not associated with maintenance of CL function during early pregnancy. However, notable changes in arachidonic acid levels of PC and PI were observed on D15 of the cycle and pregnancy. The physiological significance of the changes in this fatty acid during luteolysis and early pregnancy warrant further studies in view of the recent finding that PGF₂α invokes polyphosphoinositide hydrolysis. In addition, our results confirm the observations of Diekman et al. (1978a) that neither changes in the concentration of luteal LH receptors nor a change in their affinity corresponds to the period of maternal recognition of pregnancy in the ewe. This study provides a basic description of ovine luteal cell plasma membrane composition that could lend support to further investigations concerning plasma membrane lipid metabolism and fluidity, and their temporal relationship to the interactions between LH, PGF₂α, and PGE₂-receptor complexes with adenylate cyclase.
GENERAL DISCUSSION

Data from these experiments provided the first characterization of the lipid composition of ovine luteal cell plasma membranes collected from ewes during discrete reproductive states corresponding to luteolysis and the maternal recognition of pregnancy. Qualitative differences in total membrane phospholipids, PC, PE, SPH, PS, and PI were not observed between nonpregnant and pregnant ewes on either D13 or D15. The fatty acid compositions of PC, PE, and PS did not reveal significant differences in the relative percentages of the major fatty acids, nor in the ratio of unsaturated to saturated fatty acids. However, significant differences were observed in some of the minor component fatty acids, specifically 22:4 and 22:5. The function of these fatty acids in luteal tissue is not known. Also of interest was the tendency of 20:4 (arachidonic acid) to be decreased in PC from days 13 to 15 of the estrous cycle, while arachidonic acid of PI increased on day 15 of pregnancy. The free cholesterol:phospholipid ratios did not change with reproductive state.

These results provide support for the contention that changes in gross membrane composition are not readily associated with luteolysis or early pregnancy in the ewe. Our observations do not discount the possibility that changes in the lipid annulus associated with important
integral membrane proteins, such as the LH receptor or \( N_s \) of adenylate cyclase, may have occurred. Because polyphosphoinositide hydrolysis has been shown to be involved in the mechanism of action of PGF\(_2\alpha\) in luteal cells, the metabolism of PI and its associated fatty acids may prove to be of importance in regulating the physical properties of important plasma membrane domains during luteolysis. The precise role of PGE\(_2\), a putative "embryonic luteotropin", in regulating adenylate cyclase activity and(or) abrogating the effects of PGF\(_2\alpha\) at the level of the plasma membrane in luteal cells warrants investigation.

The results reported herein also confirm previous studies that a reduction in the concentration of unoccupied LH receptors is not involved in luteolysis. Changes in other events initiated at the plasma membrane by PGF\(_2\alpha\) appear to be more important in the initiation and completion of luteal regression.

Luteal plasma membrane changes during luteolysis and early pregnancy may be restricted to a particular cell type that would not have been detected in the present study. It would be of interest to examine this possibility by analyzing the compositions of large and small ovine cells. It would also be interesting to assess the lateral movement of the LH-receptor complex in small and large cells during different reproductive states, and to examine the possible effects of PGF\(_2\alpha\) and PGE\(_2\) on this process as it relates to membrane fluidity and adenylate
cyclase activity.

The central locus for the regulation of luteal function at the level of the luteal cell is the plasma membrane. Understanding the mechanisms underlying the control of its physical and functional properties will serve to increase our knowledge concerning the possible causes of early embryonic mortality. The goal of improving reproductive efficiency in domestic animals necessitates basic research concerning the regulation of luteal function.
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