IDENTIFICATION AND CHARACTERIZATION OF PROTEINS SECRETED BY THE CORPUS LUTEUM OF THE COW DURING THE ESTROUS CYCLE AND PREGNANCY

By

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Dedicated to Mandi, Koga, Kongwenebime, Gaston, and my mother and late father.
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Experiments were carried out to determine protein synthesis and secretion by the corpus luteum (CL) of the cow during the estrous cycle and pregnancy. Temporal changes were observed in the types of proteins synthesized across days of the estrous cycle, but not across pregnancy. The bovine CL synthesized many proteins in culture, five of which were further characterized and identified by N-terminal amino acid sequence analysis. Proteins identified were apolipoprotein E (Apo E, 35 kDa, pl 5.5), apolipoprotein A-1 (Apo A-1, 27 kDa, pl 6), tissue inhibitor of metalloproteinases-1 (TIMP-1, 30 kDa, pl 8), tissue inhibitor of metalloproteinases-2 (TIMP-2, 20 kDa, pl 8), and manganese superoxide dismutase (Mn SOD, 22 kDa, pl 8). Northern and dot

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blot analyses revealed presence of mRNA for each of the five identified proteins within bovine luteal tissue during the estrous cycle and pregnancy, confirming synthesis of these proteins by the bovine CL.

Synthesis of Apo A-1 and Apo E by the bovine CL is novel and has not been reported in any species. This is also the first report of expression of Apo A-1 mRNA by the CL. There have been reports of synthesis of TIMP-1 and TIMP-2 by the CL during the estrous cycle, but this is the first study to examine luteal synthesis of TIMP-1 and TIMP-2 during pregnancy. Similarly, this is the first report of the temporal changes in the synthesis of Mn SOD and expression of its mRNA during the estrous cycle and pregnancy in the cow.

Apolipoprotein E and Apo A-1, given their association with low density lipoprotein and high density lipoprotein, respectively, may be involved in regulating cholesterol availability for luteal membrane synthesis and steroidogenesis. TIMP-1 and TIMP-2 may be involved in steroidogenesis during the luteal phase and pregnancy, and tissue remodelling during luteal regression. Luteal Mn SOD may play a significant role in maintaining luteal function during the luteal phase and pregnancy by preventing damage of luteal cells by reactive oxygen species. These luteal proteins are believed to be acting via autocrine and/or paracrine mechanisms.
A physiological role for the ovaries was first demonstrated by Frankel (1903, cited by Short, 1977) when he showed that removal of the ovaries in rabbits resulted in termination of pregnancy. The corpus luteum of the ovary was later identified as the component responsible for maintenance of pregnancy. However, the mechanism through which the CL carries out this function was not known. Because of its high vascularity, Prenant (cited by Short, 1977) suggested that the CL is an organ of internal secretion and was capable of releasing products into systemic circulation.

The advent of new technologies and techniques such as electron microscopy made it possible to investigate the histology and biochemistry of the CL during the estrous cycle and pregnancy (Priedkaln and Weber, 1968). As a result, it is now known that the CL is composed of different types of cells; the steroidogenic small and large luteal cells, endothelial cells, macrophages, fibroblasts, and monocytes, and that there are some morphological and biochemical differences between the CL of the estrous cycle and that of pregnancy. In addition, the steroidogenic small and large luteal cells have been identified as responsible for the production of progesterone, the hormone
required for maintenance of pregnancy.

With the use of other modern research techniques in cell biology and histochemistry, it is common knowledge that luteal cells possess the structural machinery required for the synthesis and secretion of proteins. Recent research has shown that apart from the production of progesterone, the CL produces many different proteins and peptide hormones (Schams, 1989), angiogenic and mitogenic factors (Grazul-Bilska et al., 1993; Reynolds et al., 1994), and growth factors (Schams, 1989).

However, there is a paucity of information about the protein-synthetic potential of the CL across the estrous cycle and pregnancy, the nature of the proteins synthesized, factors regulating their synthesis and release, and the cell types responsible for their production. Identification and characterization of luteal proteins and factors will enable the determination of their roles in luteal growth, development and steroidogenesis, which will contribute to the understanding of fertility as well as tissue growth and development. There is an indication from some studies that other cell types, apart from the steroidogenic luteal cells, are responsible for synthesis of some luteal proteins.

Objectives of this dissertation were to examine protein synthesis by bovine luteal explants in culture during the estrous cycle and pregnancy, identify, isolate and characterize the proteins secreted, and determine presence of their respective mRNA in luteal tissue during the cycle and pregnancy.
CHAPTER 2
REVIEW OF THE LITERATURE

The Corpus Luteum

The corpus luteum (CL) is formed following the rapid development of the follicle after ovulation. Regnier de Graaf gave the first detailed description of the CL which he called "globular bodies" (Jocylyn and Setchel, 1972). Malpighi (1628-1698) introduced the term corpus luteum or "yellow body" (Short, 1977). The first attempt to define the role of the CL was made by Prenant (1898) who suggested that it is a gland of internal secretion because of its high vascularity. The CL has been shown to play several roles in the reproductive process. Most of these roles depend upon synthesis of progesterone, which is necessary for implantation, maintenance of pregnancy, control of the estrous cycle and parturition. Some of these roles were suggested following observations by Frankel in 1903 that removal of ovaries in rabbits terminated pregnancy, and this gave support to Gustav Born's hypothesis that corpora lutea are required for implantation (cited by Amoroso, 1968). If fertilization does not occur, the CL regresses in some species with a subsequent decline in circulating progesterone. The decline in progesterone concentrations leads to an increase in pulse frequency of luteinizing hormone (LH) increased follicular estrogen
synthesis, and a new wave of follicular development (Karsch et al., 1984). However, if fertilization occurs, the CL is prevented from regressing by signals sent by the embryo (Bazer et al., 1991a; 1991b). The CL could therefore be regarded as the biological clock for the events of the estrous and menstrual cycles, pregnancy and parturition.

**Histology of the Corpus Luteum**

The corpus luteum of several species is made up of different types of cells, namely, the steroidogenic large luteal and small luteal cells, endothelial cells, fibroblasts, macrophages and lymphocytes. Earlier studies (Priedikalns and Weber, 1968; Koos and Hansel, 1981) showed that the mature bovine corpus luteum contains two distinct steroidogenic cell types: the small and large luteal cells. The major differences between these two cell types were the presence of numerous large mitochondria in the large cells, and numerous lipid bodies in the small cells. Other cell types and stromal cells were also observed in the corpus luteum. The large luteal cells are larger in size (30-40 μ), with extensively folded plasma membranes which are exposed to other luteal cells and to inter-cellular vascular areas. The small luteal cells are smaller in size (15-20 μm in diameter) but with a denser chromatin pattern than the large cells. More recent studies (O'Shea et al., 1989) showed that in the bovine corpus luteum, the large luteal cells make up 3.5 % of the total cells/unit area of luteal tissue while the small luteal cells make up 26.7%, and endothelial cells /
pericytes are the most abundant (52.3%). However, large luteal cells occupy 40.2% of the corpus luteum volume/density, while the small luteal cells occupy 27.7%. Similar observations were made by Lei et al. (1991) who reported that human and bovine corpora lutea contain more nonluteal than luteal cells, and the small luteal cells are always greater in number than the large luteal cells irrespective of the reproductive states.

Ultrastructural and cytochemical studies by Parry et al. (1980) showed that large granulosa-derived bovine luteal cells are the most common cell types of the mid-luteal corpus luteum. They, however, did not observe any morphological differences between small and large cells as reported by Priedkalns et al. (1968). Large luteal cells are always close to capillaries and contain large round nuclei (10 μm in diameter), large amounts of agranular endoplasmic reticulum, and abundant mitochondria scattered throughout the cytoplasm. Bovine large luteal cells contain numerous Golgi complexes and electron-dense secretory granules during mid-cycle (Parry et al., 1980). Bovine luteal secretory granules are single membrane-bound and are 0.2-0.4 μm in diameter compared to 0.2 μm in sheep (Gemmell et al., 1974). In the cow, secretory granules are found in a cluster in the cytoplasm (granules make up 2-4% of luteal cell cytoplasm) close to the Golgi complexes, and sometimes near the edge of the cells (Parry et al., 1980). The contents of secretory granules are released by exocytosis to the intercellular space. Lipid droplets are present in some cells and concentrations varied inversely with the number of electron-
dense granules present. Morphometric analyses have shown that luteal cells occupy the maximum area of the corpus luteum on day 12 of the cycle while the protein-synthesizing compartment (ER plus polysomes and/or ribosomes) increases from day 6 to a maximum on day 17, indicating that protein synthesis is part of the metabolism of the corpus luteum during the cycle (Parry et al., 1980). The population of secretory granules is greatest on day 17 of the bovine cycle and declines thereafter indicating a drop in the secretory process, which usually precedes the fall in progesterone synthesis (Hansel et al., 1973). More recent studies in the cow have shown that unlike in sheep, luteal secretory granules are not dispersed throughout the cytoplasm but are found in a cluster (Fields et al., 1983; 1992). During the bovine cycle, the percent of large luteal cells with secretory granules is lowest on day 3 (3%), highest during mid-cycle (day 7, 84 %, day 11, 64 %) and declines on day 14 (26 %) to lowest level on days 17 (16 %) and 19 (8 %) (Fields et al., 1992). An earlier study in pregnant cows also indicated dynamic changes in the population of secretory granules during the course of pregnancy. In the large luteal cell of pregnancy, the number of secretory granules are low or undetectable prior to day 45, increases to maximum around day 200 and declines at the end of gestation (Fields et al., 1985).

There have been reports of other morphological differences between small and large luteal cells. Chegini et al. (1984) reported that large (18-45 μm) cells contain more mitochondria than small (15-18 μm) cells, and both contain
rough and smooth endoplasmic reticulum, lysosomal vesicles, Golgi complexes and membrane-bound dense granules. Granules vary in shape and form clusters close to the nucleus (Chegini et al., 1984). In a more recent study, Chegini et al. (1991) observed that the nuclear volume is greater in small than large luteal cells during estrous cycle and pregnancy. Small luteal cells are also more sensitive to hCG-induced increase in nuclear volume than the large luteal cells. The cytoplasmic:nuclear ratio is greater in large than small cells. Biochemical analysis of the two cell types have shown that the amount of protein per cell is lower in small than the large cell, while the protein/DNA ratios are similar for both. It was suggested that differences observed may have been caused by transformation of small luteal cells to large luteal cells (Chegini et al., 1984).

Biochemical Differences Between Small and Large Luteal Cells

Biochemical differences between small and large luteal cells have been reported. Small luteal cells (which make up 85% of the total luteal cell population) produce small amounts of basal progesterone and respond to secretagogues (cAMP, hCG), while the large luteal cells (which make up 8-12% of luteal cell population) produce greater amounts of basal progesterone but are not responsive to cAMP and hCG (Koos and Hansel, 1981; Fitz et al., 1982). The LH-induced increase in progesterone production by small luteal cells is mediated by an increase in cAMP. Nonhormonal activators of protein kinase A
(forskolin, cholera toxin, dibutyryl cAMP) also selectively stimulate synthesis of progesterone in small but not in large luteal cells (Wiltbank, 1994). It is not clear by what mechanism progesterone synthesis by large luteal cells is stimulated, since they have been shown to produce large amounts of progesterone in culture even in the absence of luteotropic stimuli (Wiltbank, 1994). Factors implicated in the LH-independent increase in progesterone production by large luteal cells include PGE$_2$ (Fitz et al., 1984), insulin (Sauerwein et al., 1992), and growth factors (Einspanier et al., 1990; Budnik and Mukhopadhyay, 1991; Miyamoto et al., 1992).

Alila et al. (1989) reported that treatment of bovine luteal cells with LH causes a rapid increase in intracellular free calcium in both small and large cells. Alila et al. (1989) reported that the LH-induced rise in intracellular calcium is biphasic in small cells (initial peak due to mobilization of intracellular calcium, and a second rise due to influx of extracellular calcium), while a single rise is observed in the large luteal cells. The increase in calcium is also greater for small luteal than large luteal cells (Alila et al., 1989). In an earlier study, Alila et al. (1988) observed that phorbol dibutyrate increases progesterone synthesis in bovine small, but not large luteal cells in culture. Those observations support earlier postulations that protein kinase C (PKC) is involved in progesterone synthesis in the bovine corpus luteum, and that the stimulatory effects of PKC on progesterone synthesis involves only the small luteal cells (Hansel et al., 1987). The differential response of large and small luteal cells to secretagogues seems
to be related to the distribution of receptors between the two cell types. Fitz et al. (1982) showed that small luteal cells of sheep contain 10X more LH receptors than large cells, while large cells are more enriched with prostaglandin E and F$_{2\alpha}$ receptors. PGF$_{2\alpha}$ is luteotropic in small luteal cells where it stimulates phospholipase C activity but does not reduce the LH-stimulated cAMP or progesterone accumulation (Davis et al., 1989).

Other biochemical differences between small and large luteal cells are in their abilities to synthesize and secrete proteins and peptide hormones (Schams, 1989; Rodgers, 1990). Judging from the morphology of luteal cells, large luteal cells have the intracellular organelles specialized for secretion of proteins and peptides (Anderson, 1982). Luteal tissue of sheep (Wathes and Swann, 1982), women (Wathes et al., 1982) and cows (Wathes et al., 1983; Fields et al., 1983) produces oxytocin and neurophysin. Oxytocin is present in ovine (Rodgers et al., 1983) and bovine (Fields et al., 1986; 1992) large luteal cells but not in small cells. Another peptide hormone, relaxin, is produced by corpora lutea of a multitude of mammals (see Sherwood, 1994, for review). Bagnell et al. (1989) reported that in pigs, relaxin is localized within the large luteal cell, but not the small luteal cell.
Ontogeny of Small and Large Luteal Cells

The origin of small and large luteal cells has been a matter of controversy. In the late 1800s and early 1900s, some researchers believed that granulosa cells of the ovarian follicle degenerate following ovulation, and only cells of theca developed into a corpus luteum. Others, however, thought that granulosa cells develop into a corpus luteum while thecal cells degenerate. Loeb (1906) was the first to suggest that the corpus luteum is composed of cells originating from both theca and granulosa layers. Similar observations were made in the sow (Corner, 1919), cow (Donaldson and Hansel, 1965; Lobel and Levy, 1968; Priedkalns et al., 1968), ewe (O'Shea et al., 1980), rat (Pederson, 1951) and human (Guraya, 1971). It is now believed that small luteal cells originate from theca interna of the follicle, while the large luteal cells originate from the granulosa cells. Morphological studies in sheep (O'Shea et al. 1980) and cattle (Donaldson and Hansel, 1965; Priedkalns et al., 1968) indicate that small luteal cells originate from thecal cells. Alkaline phosphatase, a marker of theca interna cells, was used to demonstrate that theca cells differentiate into small luteal cells of the ovine CL (O'Shea et al., 1980). Further support to this hypothesis was provided by observations that thecal cells incubated with forskolin and insulin for 9 days become luteinized, have low basal progesterone secretion, increased LH-induced secretion of progesterone, and do not secrete oxytocin (Meidan et al., 1990). These physiological characteristics are similar to
those exhibited by small luteal cells. Studies in sheep also suggest that
granulosa cells develop into large luteal cells because the number of granulosa
cells in pre-ovulatory follicles (O'Shea et al., 1985) approximates the number of
large luteal cells (O'Shea et al., 1986), and ovine granulosa cells undergo little
or no mitosis after ovulation (McClellan et al., 1975). Evidence for differentiation
of granulosa cells to large luteal cells was provided by observations that
incubations of bovine granulosa cells with forskolin and insulin for 9 days
resulted in luteinized cells similar to large luteal cells (high basal progesterone
secretion, reduced LH-induced progesterone secretion, and secretion of
oxytocin) (Meidan et al., 1990).

Alila and Hansel (1984) demonstrated that monoclonal antibodies
developed against theca cell membranes bound to small luteal cells while
monoclonal antibodies against granulosa cells bound specifically to the large
luteal cells. As the estrous cycle progressed, the number of large luteal cells
bound to theca antibody was similar to the number of small cells bound to theca
antibody (Alila and Hansel, 1984). They suggested that theca-derived small
luteal cells differentiate into large luteal cells as the estrous cycle progresses.
However, a study by O'Shea et al. (1986) in which comparisons of the cellular
composition of ovine luteal cells of mid- and late estrous cycle were made, did
not agree with the hypothesis that small luteal cells differentiate to large luteal
cells during the estrous cycle. Alila and Hansel (1984) also observed that large
luteal cells bound to granulosa antibody contained more mitochondria and
electron-dense granules than those bound to theca antibody. In general, the CL is a dynamic organ and its morphology changes with the reproductive state of the animal. Farin et al. (1989) reported a change in cellular composition of the ovine corpus luteum during the estrous cycle and pregnancy. The number of small cells increase with no change in size as the cycle progressed, whereas the size of large cells increased with no change in number. Thus changes in the relative proportions of the two cell types or interactions between them, may determine the function of the CL at different periods of the estrous cycle.

In sheep, the number of small steroidogenic luteal cells increases 4-fold through day 8 and then decreases through day 16 (Niswender et al., 1985). However, the number of nonsteroidogenic cells > 8 μm increases 2-fold between days 4 and 8 of the ovine cycle and declines through day 16, while the number of nonsteroidogenic cells < 8 μm reaches a peak on day 12. Because of the similarity in pattern of steroidogenic and nonsteroidogenic cells during the estrous cycle, it was speculated that small nonsteroidogenic cells are a source of stem cells that give rise to small steroidogenic luteal cells, which later develop into large luteal cells (Niswender et al., 1985).

**Intercellular Communication Among Luteal Cells**

The heterogenous nature of the cellular components of the corpus luteum is an important feature which seems to be necessary for this organ to effectively carry out its biological functions. Formation of the CL following ovulation
involves incorporation of cells from the theca and granulosa layers of the ovulating follicle. As earlier discussed, the process of luteinization involves luteal angiogenesis (Zheng et al., 1993), and an increase in size of theca and granulosa cells, and number of smooth endoplasmic reticulum and mitochondria. The corpus luteum is composed of large and small luteal cells, macrophages, monocytes, fibroblasts and endothelial cells. A substance produced by one cell may affect the function of another cell and this is referred to as cell-cell communication (Rodgers, 1990). This communication may be via gap junctions (Anderson and Little, 1984; Redmer et al., 1991) and adherens-type junctions (Weber et al., 1987; O'Shea et al., 1990) which have been observed between luteal cells. Exchange of factors among luteal cells could also occur via the blood stream (humoral). While gap junctions allow passage of very low molecular weight substances from one cell to another, adherens-type junctions serve to bind cells together (Rodgers, 1990).

Cell-Cell Communication in Luteal Angiogenesis and Development

Ovine luteal cells have been shown to produce angiogenic factors (Redmer et al., 1988; Grazul-Bilska et al., 1992). It has been proposed that growth factors such as fibroblast growth factor, insulin-like growth factor (IGF)-1, epidermal growth factor (EGF), and cytokines are involved in luteal angiogenesis (Koos, 1989). This idea was supported by observations by Zheng et al. (1993) that bovine large and small luteal cells produce basic fibroblast
growth (BFGF)-1 and -2 during the estrous cycle. BFGFs are potent angiogenic factors and their production by large and small luteal cells follow a pattern similar to luteal development (Zheng et al., 1993). Thus the steroidogenic large and small cells produce HBGFs which stimulate proliferation of endothelial cells. A functional relationship between endothelial cells and luteal cells was suggested following observations that both endothelial (Mayerhofer et al., 1992) and luteal cells (Mayerhofer et al., 1991) express a neural cell adhesion molecule. In addition, endothelial cells produce prostacyclin (PGF\(_1\)) (MacIntyre et al., 1978) which has been shown to increase concentrations of plasma progesterone in the cow (Milvae et al., 1980). A more recent study by Girsh et al. (1995) gave further evidence of interactions between endothelial cells and the steroidogenic cells of bovine CL. Endothelial cells were shown to secrete PGI\(_2\), which in turn stimulates secretion of cAMP and progesterone by bovine large and small luteal cells. However, it was also observed that the presence of endothelial cells is required for PGF\(_{2\alpha}\)-induced inhibition of progesterone production by luteal cells (Girsh et al., 1995). Thus endothelial cells of CL may regulate response of steroidogenic luteal cells to luteotropic and luteolytic signals.

IGF-I and its mRNA is produced by the bovine CL (Einspanier et al., 1990) during the estrous cycle and gestation. IGF-I has been shown to stimulate synthesis and secretion of progesterone and oxytocin by luteal tissue (Sauerwein et al., 1992). In a more recent study IGF-I was immunolocalized
mainly in bovine large luteal cells with little staining in small cells (Amselgruber et al., 1994). No IGF-I immunoreactivity was observed in pericytes, macrophages, fibroblasts or smooth muscle cells of blood vessels (Amselgruber et al., 1994). The differences in distribution of IGF-I immunoreactivity may indicate differences in cell reactivity and possible paracrine or autocrine interactions between small and large luteal cells (Amselgruber et al., 1994). A role of IGF-I in CL function has been suggested following observations that concentrations of luteal IGF-I increase during early and mid-luteal phases and decline rapidly after luteolysis (Einspanier et al., 1990).

**Cell-Cell Interaction in Luteal Hormone Synthesis**

Luteal progesterone is produced mainly by the large luteal cells in most species. However, progesterone production by isolated populations of porcine small and large luteal cells has been shown to be greater when both cell populations are cultured together than when cultured separately (Lemon and Mauleon, 1982). The increase in progesterone production observed in cocultures was attributed to stimulation of progesterone production in large cells by some factor(s) produced by small cells, suggesting an interaction between small and large luteal cells. Oxytocin, a peptide hormone produced by CL of ruminants is thought to be involved in cell-cell interaction between small and large cells because it is produced only by large luteal cells (Fields and Fields, 1986; Theodosis et al., 1986) and is capable of inhibiting LH-induced $P_4$.
production by small luteal cells (Schams, 1989).

**Corpus Luteum of the Estrous Cycle**

**Luteinization and CL Development**

Corpora lutea are formed following ovulation of a mature follicle mediated by gonadotrophin stimulation. Following the preovulatory LH surge, a series of morphological and biochemical changes take place within the follicle to change the latter to a corpus luteum. The LH surge serves dual roles of stimulating ovulation and converting the follicle to a corpus luteum. This luteinization process involves breakdown of the basement membrane between theca and granulosa layers, invasion of the follicular antrum space by blood vessels, and development of an extensive vascular network (Zheng et al., 1993; Niswender et al., 1994). The invading capillaries are formed via both migration and mitosis of endothelial cells (Zheng et al., 1993). After ovulation, the follicle grows rapidly to 10 times its weight in just 7 days. This increased growth is attributed to hypertrophy and hyperplasia of thecal cells which migrate into the hollow follicular antrum after ovulation, and integrate among luteinizing granulosa cells (O'Shea et al., 1980). Formation of the CL is initiated by a series of morphological and biochemical changes in the theca and granulosa cells of the preovulatory follicle. This process, referred to as luteinization, changes the follicle from a predominantly estradiol-producing structure to one that secretes
progesterone.

Morphological changes associated with luteinization include accumulation of smooth endoplasmic reticulum, mitochondria with tubular cristae, increase in size of Golgi apparatus, and accumulation of glycogen-containing granules (Niswender and Nett, 1994). These changes provide the CL with the ability to efficiently produce progesterone. During the luteinization process, theca and granulosa cells of the preovulatory follicle differentiate into small and large luteal cells, respectively (Meidan et al., 1990). Other morphological changes associated with luteinization include an increase in the cytoplasmic nuclear ratio and appearance of a large number of lipid droplets containing sterol esters. After ovulation, there is an increase in gap junctions among developing luteal cells in rats (Anderson and Little, 1984). O'Shea et al. (1990) reported the presence of adherens-type junctions between small and large luteal cells of cattle. In a more recent study, Redmer et al. (1991) reported the presence of gap junction-like structures in bovine luteal cells from mid-cycle.

Biochemical changes associated with luteinization of the follicle include a switch from a predominantly estradiol-producing structure to one that secretes mainly progesterone. During luteal formation, there is an increase in expression of mRNA and enzyme activity for \( P_{450} \) side chain cleavage and 3\( \beta \)-hydroxysteroid dehydrogenase (3\( \beta \)HSD). There is also an increase in activity of cholesterol esterase as the CL becomes fully functional. These changes are consistent with the CL's role in progesterone synthesis. In contrast, luteinization decreases
estrogen production by decreasing levels of mRNA and protein for 17α-
hydroxylase cytochrome P₄₅₀, the enzyme that catalyses conversion of
pregnenolone or progesterone to androgen. In preovulatory follicles in cattle
(Rodgers et al., 1987) and rat (Hedin et al., 1987), the expression of mRNA and
protein levels for aromatase cytochrome P₄₅₀ enzyme also decreases rapidly
after the LH surge. Corpora lutea of the rat express aromatase mRNA and
produce estradiol, while CL of domestic ruminants do not synthesize estradiol
(Savard, 1973). Other biochemical changes observed after ovulation include
reduced expression of genes encoding FSH and LH receptors in granulosa
follicular cells, which results in a down regulation of both receptors. In contrast,
LH receptor levels increase with formation of the CL in ewes (Diekmann et al.,
1978). In support of this observation in rat, expression of the gene encoding the
LH receptor increases with development of the CL (Segaloff et al., 1990).

Luteal Angiogenesis

Angiogenesis is one of the features of luteinization, and continues after
formation of the CL. Blood flow to the CL increases with luteinization and
accounts for about 90% of the total ovarian blood flow during the mid-luteal
phase. At this time, about 60% of each luteal cell’s surface directly faces a
capillary (Keyes and Wiltbank, 1988). In the rat, luteal blood flow and the
number of luteal endothelial cells increase during mid-pregnancy (Bruce et al.,
1984). The ability of the CL to cause angiogenesis was first reported by Jakob
et al. (1977). Corpora lutea of sheep and cattle have been shown to produce angiogenic factors in culture (Redmer et al., 1988). Gospodarowicz et al. (1985) also isolated an angiogenic factor from the bovine CL which accounted for 84% of the angiogenic activity in crude CL extracts, and had amino acid sequence homology with bovine brain and pituitary fibroblast growth factor. It is not clear what regulates angiogenesis or the high rate of blood flow to the CL. However, several factors which affect endothelial cell proliferation are proposed as regulators of luteal angiogenesis. These include prostaglandin E, epidermal growth factor (EGF), endothelial growth factor, endothelium-stimulating factor, angiogenin, insulin and transferrin (Findlay, 1986).

Development of luteal vasculature is a dynamic process which varies with the stage of the estrous cycle. Zheng et al. (1993) observed that capillary density within luteal tissue is sparse in the early luteal phase (days 1-4 post ovulation), high in the middle phase (days 5-17), and is reduced dramatically in the late phase (days 18-21). Since a reduction in tissue function is usually associated with a decline in blood flow and vascularity, the fall in blood flow and vascularity during the late luteal phase could indicate degeneration of luteal cells and a decline in luteal function (Zheng et al., 1993). Redmer et al. (1988) observed that luteal-conditioned medium from early (days 1-4), mid (days 5-17), and late (days 18-21) ovine cycle stimulates angiogenesis (mitogenesis and migration of endothelial cells. Angiogenic activity increases with advancement of the luteal phase. In that study, LH stimulated the production and/or release of
the angiogenic factors, while PGF$_{2\alpha}$ blocked the LH-induced stimulation (Redmer et al., 1988). Thus, luteal vasculature could be stimulated in an autocrine/paracrine manner by angiogenic factors produced by the corpus luteum.

It has also been suggested that growth factors may regulate ovarian angiogenesis since they are present in the ovary, and have effects on endothelial cells. Koos (1989) speculated on the possible roles of fibroblast growth factor, insulin-like growth factors (IGFs), EGF, TGF$\alpha$ and TGF$\beta$ in ovarian angiogenesis. Tumor necrosis factor alpha (TNF$\alpha$), PGE$_1$, PGE$_2$, estradiol, plasminogen activator proteolytic enzymes (plasminogen activator, plasmin, collagenase) also stimulate angiogenesis. However, it is not clear how the expression and activities of these factors are regulated, and how they modulate luteal angiogenesis. In a recent study by Zheng et al. (1993), pattern of immunostaining for heparin binding growth factor (HBGF) (also known as basic FGF) in bovine luteal tissue was parallel to that of luteal vascular development throughout the estrous cycle, suggesting a role of HBGF in vascular development. Follicular granulosa and theca interna cells, macrophages, endothelial cells, and mast cells have been implicated as involved in the regulation of angiogenesis in the ovary and the CL (Koos, 1989). Brannstrom and Norman (1993) proposed that mast cells present in CL of some species may modulate the luteinization process by producing and secreting cytokines and proteases involved in tissue remodelling, angiogenesis and
stimulation of progesterone production.

Mechanisms involved in the control of blood flow to the CL are not known and are still hypothetical. Studies in the rabbit led to rejection of the hypothesis that luteotropic hormones promote luteal blood flow (Keyes and Wiltbank, 1988). Rather, it was suggested that blood flow to the CL is not regulated by luteotropic hormones, and has no correlation with the level of luteal steroidogenesis.

**Regulation of Luteal Function During the Estrous Cycle**

With its formation, the CL is composed of mainly steroidogenic small and large luteal cells, and endothelial cells, pericytes, macrophages, smooth muscle cells and fibroblasts. The number, volume and density of small and large luteal cells vary throughout the estrous cycle, but volume of CL occupied by each cell type stays relatively constant (Niswender et al., 1994). Progesterone is the major hormone produced by the CL during the luteal phase of the cycle and LH is the major luteotropin that stimulates luteal progesterone production in several species.

The luteotropic regulation of the CL has been the subject of much research. Corpus luteum formation is thought to be induced by the preovulatory LH surge. Dependence of the CL on LH has been tested in several studies involving hypophysectomy, administration of LH antibodies, and administration of GnRH antagonists. In sheep, hypophysectomy 5 days post estrus does not affect serum and luteal progesterone concentrations, although CL weight on day
12 was lower than expected (Farin et al., 1990). These results indicated that a CL deprived of LH may still function although its growth and development could be compromised. Baird (1992) observed that most LH pulses on days 6-7 and 13-14 of the ovine cycle are followed by a rise in progesterone concentration. However, changes occur in concentrations of progesterone independent of LH pulses. Injection of a GnRH antagonist during early luteal phase also causes a small decline in progesterone production, whereas administration of GnRH antagonist on day 13 causes a rapid decline in progesterone secretion, and luteal regression (Baird, 1992).

Thus the CL requires a luteotropic support from LH during early and mid-luteal phases of the cycle. However, the CL of mid-cycle seems to be less resistant to withdrawal of luteotropic support than the early luteal phase CL. It was suggested that high levels of progesterone during the mid-luteal phase cause a reduction in frequency of endogenous LH pulses. Also, uterine PGF$_{2\alpha}$, which is secreted during the mid-luteal phase, may interfere with coupling of LH to the adenyl cyclase second messenger system, and increase sensitivity of the CL to PGF$_{2\alpha}$ as a result of the long intervals between LH pulses. These events would result in luteal regression (Baird, 1992).
Luteal Regression

When the mature follicle is not fertilized, the CL is eventually destroyed thereby allowing the female another opportunity to start a new cycle, ovulate and become pregnant. Destruction of the CL is referred to as luteolysis or luteal regression. The basic features of luteolysis are a decline in progesterone secretion which is referred to as functional luteolysis, followed by structural changes which lead to breakdown of luteal cells. The drop in circulating progesterone reduces the negative feedback of progesterone on the pituitary and leads to an increase in gonadotropin pulse frequency, a new wave of follicular growth, and ovulation.

Role of PGF$_{2\alpha}$ in Luteal Regression

The mechanism of luteal regression has been the subject of numerous studies. Prostaglandin F$_{2\alpha}$ from the uterus is the natural luteolysin in domestic farm animals and most rodents (McCracken et al., 1972; Knickerbocker et al., 1988). Due to the close apposition of the uterine and ovarian blood vessels, there is a local transport of PGF$_{2\alpha}$ from the uterine vein to the ovarian artery, making it possible for PGF$_{2\alpha}$ to reach the CL without going through systemic circulation (Del Campo and Ginther, 1973). The luteolytic effect of PGF$_{2\alpha}$ occurs through its interaction with ovarian oxytocin. The pulsatile release of oxytocin from the CL stimulates the pulsatile release of PGF$_{2\alpha}$ from the uterus, which in turn positively feeds back to further increase luteal oxytocin release. This
positive feedback loop continues until the demise of the corpus luteum (Jenkin, 1992a).

The endometrial oxytocin receptor is thought to be the determining factor as to whether or not luteolysis will occur (Flint et al., 1992a). Inhibition of uterine oxytocin with a synthetic oxytocin receptor antagonist prevents pulsatile release of PGF$_{2\alpha}$, and luteolysis (Jenkin, 1992a). It is believed that trophoblast interferons in ruminants prevent luteal regression during early pregnancy via a similar mechanism (Flint et al., 1992; Jenkin, 1992b). This local effect of PGF$_{2\alpha}$ is not present in horses because of a different uterine-ovarian vascular anatomy and the equine CL will regress even in the absence of the ipsilateral uterine horn. In contrast to the horse, removal of the uterine horn ipsilateral to the CL in ruminants, pigs and some rodents prevents luteal regression, whereas removal of the contralateral horn has no effect on luteal lifespan (Ginther, 1974).

Fairclough et al. (1981) reported that passive immunization of cows and ewes with PGF antibodies prolonged the estrous cycle, demonstrating PGF$_{2\alpha}$'s luteolytic function. Copelin et al. (1989) reported that cows actively immunized against PGF$_{2\alpha}$ exhibit prolonged luteal lifespan after first ovulation. Cows with higher PGF$_{2\alpha}$ antibody titres had longer luteal lifespan and progesterone secretion (Copelin et al., 1989). In a more recent study, active immunization of ewes against PGF$_{2\alpha}$ on day 5 postpartum prevented ovulation (Bettencourt et al., 1993). However, in rhesus monkeys, humans and dogs, the uterus does not seem to be needed for luteal regression (Neill et al., 1969), and an intraluteal
production of PGF$_{2\alpha}$ has been suggested in these species (Niswender et al., 1994).

Several mechanisms have been proposed by which PGF$_{2\alpha}$ exerts its negative effects on luteal function. Nett et al. (1976) proposed that PGF$_{2\alpha}$ causes degeneration of luteal cells by causing a reduction in blood flow leading to hypoxia within luteal tissue. Nett et al. (1976) observed degeneration of capillary endothelial cells during luteal regression. It has been suggested that the decrease in blood flow could be due to a degeneration of luteal capillaries rather than vasoconstrictive effects of PGF$_2\alpha$ (Wiltbank et al., 1990). The changes in luteal vasculature observed across the estrous cycle correlated with luteal growth, development, and regression (Zheng et al., 1993), and supports earlier reports on the role of blood flow in the control of luteal regression.

Capillary density was low in the early luteal phase (days 1-4), high in mid-cycle (days 5-17), and decreased dramatically in the late stage (days 18-21). Like in cattle, PGF$_{2\alpha}$ rapidly decreases luteal blood flow in ewes with a corresponding decline in circulating progesterone.

It has been proposed that PGF$_{2\alpha}$ decreases LH binding to luteal cells in vivo and may block stimulation of adenylate cyclase by LH (Niswender and Nett, 1994). In vitro, PGF$_{2\alpha}$ has also been shown to block the LH-induced increase in cAMP and progesterone production by ovine luteal tissue (Niswender and Nett, 1994). Wiltbank and Niswender (1992) proposed that the luteolytic action of PGF$_{2\alpha}$ involves binding of PGF$_{2\alpha}$ to a specific membrane receptor on large luteal
cells, activation of phosphoinositide-specific phospholipase C, and an increase in intracellular calcium which activates protein kinase C (PKC). PKC inhibits intracellular cholesterol transport leading to a decrease in progesterone production (Wiltbank and Niswender, 1992). Thus the antisteroidogenic effects of PGF$_{2\alpha}$ are mediated through the PKC second messenger system (Niswender et al., 1994). The sustained increase in free intracellular calcium causes degeneration and death of large luteal cells (Wiltbank et al., 1989).

It has also been proposed that the antisteroidogenic effects of PGF$_{2\alpha}$ on the CL could be due to a reduction in the number of LH receptors (Behrman et al., 1978), and the uncoupling of the LH receptors from the adenylate cyclase second messenger system (Fletcher and Niswender, 1982). It has been shown that treatment of luteal cells with PGF$_{2\alpha}$ inhibits formation of cAMP by LH in vitro (Dorflinger et al., 1983).

Apart from intracellular changes, the CL undergoes morphological changes during regression. The plasma membrane of the regressing CL contains gap junctions, maculae adherens, coated invaginations and microvilli (Niswender and Nett, 1994). In the bovine CL, a decrease in amount of smooth ER, an increase in number of autophagic vacuoles and an increase in number of lipid droplets in cytoplasm are also observed during regression (Fields et al., 1992). Other morphological changes observed in regressing bovine CL are a decrease in the number of secretory granules, presence of numerous swollen mitochondria, and a decrease in size of the steroidogenic cells (Niswender and
These morphological changes are common to several species. The increase in lipid droplets and cytoplasmic vacuoles have been observed in guinea pigs (Paavola, 1979), humans (Vanlennys and Madden, 1965) and rabbits (Koering and Thor, 1978).

These observations led to suggestions that the immune system may play a role in luteal regression. Bovine luteal cells have been shown to express MHC class II antigens and their expression increase with advancing age of the corpus luteum (Pate, 1994). Expression of MHC class II antigens is restricted to the large luteal cells during mid-cycle, but is observed in both the small and large luteal cells prior to luteal regression (Benyo et al., 1991). Luteal MHC II antigen expression has also been observed during PGF$_{2\alpha}$-induced luteolysis, but expression is absent in pregnant cows (Benyo et al., 1991). Interferon-gamma has also been shown to induce expression of MHC II in bovine luteal cells (Fairchild and Pate, 1989). Thus IFN-gamma may contribute to the luteolytic process by stimulating luteal prostaglandin synthesis and inhibiting progesterone production (Pate, 1994).

**Role of Oxygen Free Radicals in Luteal Regression**

The role of oxygen free radicals in the luteolytic process has been the subject of recent studies. In the rat, luteal levels of superoxide anions and hydrogen peroxide increase after treatment with PGF$_{2\alpha}$, and during regression (Sawada and Carlson, 1989). It has been suggested that oxygen radicals may cause lipid peroxidation, which in turn, stimulates luteal PGF$_{2\alpha}$ production, which
may contribute to luteolysis (Carlson et al., 1993). However, the mechanism by which superoxide radicals inhibit progesterone secretion in vivo is not known, although superoxide radicals have been shown to inhibit luteinizing hormone (LH) stimulation of cAMP, and cAMP-induced progesterone secretion in rat luteal cell cultures (Gatzuli et al., 1991). Superoxide dismutase, the enzyme that converts superoxide anions to hydrogen peroxide, increases as the luteal phase progresses and LH has been shown to induce its synthesis (Laloraya et al., 1988). These results suggest that oxygen radicals are involved in the luteolytic process since their removal favors synthesis of progesterone, as opposed to the decline in progesterone observed during luteal regression.

**Corpus Luteum of Pregnancy**

When fertilization occurs following ovulation, the CL does not regress but rather is maintained and becomes responsible for producing progesterone required to maintain pregnancy. In the cow, the CL is the major source of circulating progesterone during most of pregnancy. Later in pregnancy, the placenta can adequately provide progesterone. Thus in the cow, the CL is not required as a source of progesterone after day 200 of pregnancy. However in the pig (Nara et al., 1982) and the rat (Steinetz et al., 1976), the CL is required throughout pregnancy.
CL Morphology During Pregnancy

Morphologically, the CL of pregnancy is similar to that of the estrous cycle (Fields et al., 1985). It is made up of steroidogenic large and small luteal cells, macrophages, lymphocytes, endothelial cells, fibroblasts, as discussed earlier. However, Weber et al. (1987) observed some morphological differences between steroidogenic cells of the cycle and those of pregnancy. Viability of small luteal cells is significantly higher in cyclic than in pregnant cows, while viability of large cells is not different between estrous cycle and pregnancy (Weber et al., 1987). The significance of these observations is not known.

Biochemically, the CL of pregnancy differs from that of the cycle. Luteal cells of late pregnancy produce less progesterone than those of nonpregnant cows (Fields et al., 1985). Weber et al. (1987) observed that large luteal cells of pregnant cows produce 30 times less progesterone than those of nonpregnant cows. It has also been shown that small luteal cells of pregnant cows are unresponsive to exogenous LH in contrast to the small luteal cells of nonpregnant cows (Weber et al., 1987). More recent studies indicate differences in protein synthesis and secretion between luteal cells of pregnancy and the estrous cycle. In the cow, oxytocin and neurophysin are localized within secretory granules of large luteal cells during the cycle, but are absent in luteal secretory granules after day 40 of pregnancy (Fields et al., 1992).

When pregnancy occurs, biochemical communications take place
between the conceptus and the mother to prevent regression of the corpus luteum, and thus sustain production of progesterone required to maintain pregnancy. This phenomenon is referred to as maternal recognition of pregnancy, whereby the conceptus sends signals to the maternal system to prevent regression of the corpus luteum (Short, 1969). The strategies used for maternal recognition of pregnancy vary among species and involve different proteins.

**Maternal Recognition of Pregnancy**

In cattle, luteolysis is prevented during pregnancy by inhibition of both basal and oxytocin- or estradiol-stimulated PGF$_{2\alpha}$ secretion via synthesis of an endometrial prostaglandin synthase inhibitor (Thatcher et al., 1992). In the cow, presence of the CL is required for maintenance of pregnancy through the first 200 days of gestation. Ovariection of pregnant cows prior to day 200 results in abortions (Estergreen et al., 1967). The bovine placenta does not contribute significantly to circulating progesterone concentrations even after day 200 of gestation.

Maternal recognition of pregnancy in cattle occurs on days 16-17 postestru (Niswender and Nett, 1994). At this time, the conceptus secretes embryonic interferons (interferon-tau) which act via endometrial receptors and alter the secretion of endometrial PGF$_{2\alpha}$ (Roberts et al., 1992). Earlier studies have shown that high amplitude pulses of PGF$_{2\alpha}$ occur in nonpregnant heifers
during luteolysis, but are absent in pregnant ones (Kindahl et al., 1976). Similar observations have been reported in ewes (Zarco et al., 1988) and buffaloes (Batra and Pandey, 1983). Following the transfer of day 15 or 16 bovine embryos to recipients, recipients with regressed CL were observed to have four to five spikes of PGFM, whereas recipients in which the CL persisted had reduced or no PGFM spikes (Betteridge et al., 1984). PGF$_{2\alpha}$ is the uterine luteolysin in cows. Thatcher et al. (1985) observed that the estradiol-induced increase in PGFM production is inhibited by the presence of a conceptus on day 18 of pregnancy, but is not in cyclic cows on day 18 of the estrous cycle. The conceptus provides greater inhibition of PGFM production on day 20 than the day 18 conceptus because the former has a more extensive contact with the endometrium (Thatcher et al., 1985). Similarly, the oxytocin-induced increase in uterine PGFM is significantly less in pregnant than in nonpregnant heifers on day 19 postestrus (LaFrance and Goff, 1985). These studies suggested an antiluteolytic-antiPGF effect of the conceptus.

The conceptus mediates its antiluteolytic-antiPGF effect via the secretion of proteins. Cyclic cows receiving intrauterine injections containing secretory proteins found in days 16-18 conceptus-conditioned medium have longer interestrus intervals than those cows receiving serum proteins (Thatcher et al., 1985). Intrauterine injections of bovine conceptus proteins also reduce estradiol-stimulated PGFM production (Bazer et al., 1986). The major proteins secreted by cultured bovine conceptuses have molecular weights of 22-26 kDa.
and isoelectric points of 5.6-5.8. The protein is referred to as interferon-tau and it shares 50% amino acid sequence identity with recombinant bovine interferon-alpha (rbIFNα) (Imakawa et al., 1989). Bovine trophoblast protein-1 given at time of maternal recognition of pregnancy extends luteal function in the cow (Thatcher et al., 1989) and ewe (Parkinson et al., 1992). Similarly, intrauterine infusion of recombinant bovine interferon-α extends the length of the estrous cycle in post-partum cows expected to have short luteal lifespan (Garverick et al., 1992). The recombinant interferon acts by reducing oxytocin-induced PGFM release (Plante et al., 1990). Treatment of ewes with recombinant bovine interferon-alpha I on days 9-19 post-estrus caused a reduction in plasma concentrations of PGFM when compared to control groups (Parkinson et al., 1992). Bovine trophoblast protein-1 exerts its antiluteolytic effects by inhibiting synthesis and/or recycling of endometrial oxytocin receptors directly, or by inducing synthesis of a PGF₂α synthase inhibitor (Bazer et al., 1991). However, the antiluteolytic signals do not seem to act directly on the CL (Bazer et al., 1991). Bovine conceptuses also produce PGE₂ and small amounts of estradiol which together stimulate increase in uterine blood flow which may enhance delivery of antiluteolytic-luteoprotective agents to the ovary (Lewis et al., 1982; Thatcher et al., 1986).

In the ewe, maternal recognition of pregnancy occurs on days 12-13 post-estrus. Although serum progesterone concentrations are similar between pregnant and nonpregnant ewes at this time, the conceptus prevents regression
of the CL in pregnant ewes (Niswender and Nett, 1994). During the ovine cycle, prostaglandin F$_{2\alpha}$ is produced by the endometrium and transported to the ovary where it causes regression of the CL and a decline in progesterone production. Between days 12-21 of gestation, trophoblast cells of ovine blastocysts secrete proteins including a major secretory 17 kDa protein (interferon-tau). IFN-tau is not produced beyond day 21 of gestation, and is the only secretory product detected on day 13 (Godkin et al., 1982). Intrauterine infusion of IFN-tau on days 12-18 of the estrous cycle extends corpus luteum lifespan in ewes, while the CL regresses in untreated ewes. It was also observed that ewes treated with sheep serum ovulated and formed a new CL while ewes treated with total conceptus proteins did not ovulate (Godkin et al., 1984). Fincher et al. (1984) showed that IFN-tau inhibits estradiol- and oxytocin-induced uterine production of PGF$_{2\alpha}$. Thus IFN-tau is antiluteolytic and anti-PGF. Oxytocin-induced PGF production is lower in pregnant than in nonpregnant ewes (Fairclough et al., 1984).

The mechanism(s) of action of IFN-tau is not clear but seems to be via its binding to endometrial receptors (Godkin et al., 1984; Hansen et al., 1989), causing changes in the secretion of endometrial proteins and prostaglandins, and extending luteal function (Vallet et al., 1988). Treatment of ewes with ovine conceptus secretory proteins (oCSP) on days 11-15 post-estrus causes a decline in concentrations of endometrial estrogen receptors, estrogen receptor mRNA, and progesterone receptor on day 16 when compared with ewes treated
with serum proteins. Ovine conceptus secretory proteins also reduce oxytocin binding and activation of the phosphoinositol second messenger system (Mirando et al., 1993). In another study, maximum expression of endometrial progesterone receptor mRNA occurred earlier (days 10-12 post-estrus vs days 14-16) in pregnant than in cyclic ewes, and oxytocin stimulated in vitro endometrial production of inositol phosphates in cyclic but not in pregnant ewes (Ott et al., 1993).

Nephew et al. (1989) have shown that intramuscular injections of recombinant bovine interferon-tau cause an increase in pregnancy rate, prolificacy and higher survival of conceptuses in ewes. Also, plasma concentrations of PGE\textsubscript{2} in utero-ovarian vein in ewes increase during maternal recognition of pregnancy (Silvia et al., 1984). IFN-tau and other conceptus proteins also favor production of PGE\textsubscript{2} over PGF\textsubscript{2a} and thus prevent luteal regression. Ovine IFN-tau, like bovine IFN-tau, acts by binding to endometrial receptors (Godkin et al., 1984; Hansen et al., 1989), changes secretory patterns of endometrial proteins and prostaglandins, and extends luteal function (Vallet et al., 1988). Intrauterine infusions of oCSP and bovine recombinant interferon-alpha 1 on days 12, 13 and 14 of the estrous cycle cause a decline in concentrations of endometrial oxytocin receptor, and the oxytocin-induced increase in PGFM (Vallet and Lamming, 1991).

Ovine IFN-tau genes are expressed specifically by cells of the trophectoderm and are regulated in a developmental manner. Expression of the
gene is present in day 10-11 blastocysts, increases by day 13, declines slightly by day 15 and sharply thereafter (Guillomot et al., 1990). Roberts et al. (1992) have also shown that IFN-tau gene expression is undetectable by day 22, a time when most of the trophoblast is attached to the uterine epithelium. The amount of protein produced by the blastocysts correlated with the expression of oIFN-tau mRNA (Roberts et al., 1992). Although oIFN-tau gene expression is the amount developmentally regulated but other factors may affect its production. In sheep and cattle, an advanced luteal phase enhances conceptus development and earlier expression of IFN-tau (Garrett et al., 1988; Nephew et al., 1991). Xavier et al (1991) observed a simultaneous expression of c-fos proto-oncogenes and IFN-tau in ovine trophoblasts. Also in the pig, endometrial expression of c-fos mRNA increases on day 12 of pregnancy (day of maternal recognition of pregnancy) and is higher when compared to expression on day 12 of the cycle (Dubois et al., 1993). Thus c-fos may be induced by IFN-tau or may be involved in the transcriptional activation of IFN-tau genes. However, no interferon response elements are present on the c-fos gene, and no AP-1 binding sites have been found in the promoter region of IFN-tau genes. It is also possible that both c-fos and IFN-tau genes are regulated by common mechanisms during the period of maternal recognition of pregnancy (Roberts et al., 1992).
Protein Synthesis by the Corpus Luteum

Apart from its role in the synthesis of progesterone required for the maintenance of pregnancy and control of the estrous cycle, the CL has been shown to produce a number of proteins, peptide hormones and factors. However, most of the proteins have not been fully characterized and attempts to define their functions have been mainly speculative.

The CL produces oxytocin, neurophysin, relaxin, inhibin, vasopressin, \( \beta \) endorphin, growth factors, angiogenic factors and protease inhibitors. Results in this dissertation have shown that the CL also produces many proteins including apolipoproteins E and A-1, tissue inhibitor of metalloproteinases-1 and 2, and manganese superoxide dismutase (see table 1-1). The types of proteins produced by the CL vary with species and the reproductive state of the animal. Relaxin is produced by CL of human, pig, rat, but not by CL of ruminant species (Sherwood, 1994). On the other hand, oxytocin and neurophysin are produced by CL of ruminants but not by nonruminants. These proteins carry out different functions in the CL, the ovary, and at extraovarian sites. The chemical nature and the physiological roles of some of these proteins have not been fully defined.

Luteal proteins and factors are produced by different cell types within the CL. Cell-cell communication takes place in order to co-ordinate the functions of luteal cells, and regulate synthesis and release of proteins. Factors regulating
synthesis, release, and biological activity of luteal proteins/factors are not fully understood.

**General Overview of Protein Synthesis and Release**

In order to produce a protein, a cell must possess the genetic material (gene) that codes for that specific protein. The DNA template directs the synthesis of RNAs which are involved in protein synthesis. Evidence for RNA involvement in protein synthesis was reported in 1930 when it was observed that a crude preparation of RNA was rich in protein, and the concentration of RNA-protein particles (ribosomes) correlated with the rate of protein synthesis by the cell. Francis Crick in 1958 defined the relationship between DNA, RNA and protein (Crick, 1970). Proteins are synthesized on ribosomes from specific mRNAs. The mRNA specifies the protein to be synthesized, and associates with the ribosomes to initiate the process. More recently, Xing et al. (1993) showed that mRNAs are produced at specific locations in the nucleus and are then exported to the protein synthesizing machinery in the cytoplasm. RNA metabolism is also organized in the nucleus in association with the nuclear matrix (Carter et al., 1993). It has been suggested that the nuclear matrix may determine what genes are turned on by sequestering and concentrating the DNA to be transcribed, as well as the transcription factors necessary for a specific gene expression. Ribosomes associate with transfer RNA and move along the mRNA to form peptide bonds.
Protein Secretion

Most cells have the ability to secrete proteins. Proteins are secreted via either a regulatory or a constitutive pathway. Secretion of most eukaryotic proteins require their transport across the endoplasmic reticulum (ER) membrane. This process occurs in two steps; targeting, followed by active transfer across the ER membrane (Rapoport, 1992).

Protein Targeting

Secretory proteins synthesized on ribosomes in the cytoplasm are targeted to the ER membrane by signal sequences. The signal sequence is targeted to the ER membrane by the Signal Recognition Particle (SRP). The nascent peptide chain-ribosome-SRP complex binds to the ER membrane by an interaction with the membrane-bound SRP receptor or docking protein. Guanosine triphosphate (GTP) hydrolysis causes the SRP to detach from the ribosome and signal sequence, then the nascent peptide is transferred to the ER membrane while the ribosome stays bound to the membrane via a ribosome receptor. Finally, a GTP hydrolysis reaction causes the SRP to dissociate from its receptor, and a new targeting cycle can begin (Rapoport, 1992).
Translocation Across ER Membrane

Proteins targeted to the ER membrane are transported across the membrane at specific sites. The translocation site is a complex and dynamic structure composed of many proteins and enzymes that catalyze modification of nascent peptides. Although proteins seem to be translocated through protein-conducting channels, the mechanism is not clear (Rapoport, 1992). Most secretory proteins are translocated as precursors with larger masses than the final mature protein. The mature protein is obtained after cleavage of the signal sequence during passage across the ER membrane and the Golgi apparatus.

Processing and Sorting of Proteins in the Golgi

After transversing the ER, the protein is transported in vesicles to the Golgi complex via an energy-dependent process. In the Golgi, the protein may be post-translationally modified by attachment of functional groups (glycosylation, acetylation, sulfation). The protein is either concentrated in secretory granules and released in response to an appropriate stimulus (regulatory pathway) or is transported to the cell surface in a vesicle and released directly (constitutive pathway). Exocytosis involves an interaction between proteins on the cytoplasmic surface of secretory granules (vesicles) and proteins of the inner surface of the plasma membrane (Widnell and Pfenninger, 1990).
One of the objectives of this research was to identify proteins synthesized and released in culture by the bovine CL during the estrous cycle and pregnancy. Five of the proteins were identified by N-terminal amino acid sequence analysis. The next section of this chapter discusses the identified proteins.

Apolipoprotein A-1

Biochemical Characterization

Apolipoprotein A-1 (Apo A-1) is the major protein component of high density lipoprotein (HDL), and accounts for 80% or more of the protein moiety of HDL in the cow and all mammalian species (Sparrow et al., 1992). Apolipoprotein A-1 has metabolic and structural roles since it contributes to the size and shape of the lipoprotein particle, solubilizes water-insoluble lipids, and is a potent activator of lecithin:cholesterol acyltransferase (LCAT). Apolipoprotein A-1 is also involved in the recognition and modulation of enzymes involved in lipid metabolism, and binding of lipoproteins to their cellular receptors (Hopkins et al., 1986).
Apo A-1 gene

Mammalian Apo A-1 gene shows no striking region of evolutionary conservation, and the bovine Apo A-1 gene is more closely related phylogenetically to canine than to human and other mammalian lineages (O'hUigin et al., 1990). The cDNA of Apo A-1 cloned from a bovine cDNA library (longest insert 963 nucleotides) was shown to contain an open reading frame of 795 nucleotides flanked by 72 and 96 nucleotides at the 5' and 3' end, respectively (O'hUigin et al., 1990). The 3' flanking region contains a polyadenylation signal (AATAAA) 14 nucleotides upstream of a poly-A tail. Based on the cDNA sequence the derived amino acid sequence contains an 18-residue signal peptide and a 6-residue prosegment.

Metabolism

Apo A-1 is synthesized as a prepro-Apo A-1 mainly by the liver and intestine, but is also synthesized by other peripheral tissues such as kidney, adrenal, and testis (Blue et al., 1982), and brain endothelial cells (Guttler et al., 1990). Sorci-Thomas et al. (1988) showed that the liver and small intestine contribute to most of plasma Apo A-1, and suggested that other tissues observed to synthesize Apo A-1 may not contribute significantly to the plasma Apo A-1 pool, but may play a role in lipid metabolism within these tissues in an autocrine and/or paracrine manner.
The primary structure of Apo A-1 varies with species. Bovine Apo A-1 is composed of 241 amino acid residues. The propeptide has a sequence (Arg-His-Phe-Trp-Gln-Gln), and approximately 10% of bovine plasma Apo A-1 is in the propeptide form (Sparrow et al., 1992). Newly-synthesized Apo A-1 from different tissues exists in four isoforms (two major and two minor) with isoelectric points ranging between 5.3 and 5.7, similar to Apo A-1 from the liver (Blue et al., 1982). The nucleotide and deduced amino acid sequence of bovine Apo A-1 shares 80% homology with the human and rabbit sequences (Gu et al., 1993). The central region of bovine Apo A-1 is hydrophobic, with highly hydrophilic regions at the amino and carboxy termini (O'hUigin et al., 1990). The hydrophobic amphipathic helical regions are necessary for interaction of apoprotein with phospholipid-cholesterol complexes (Sparrow et al., 1992).

Bovine Apo A-1 contains a single methionine and no cysteine as do the canine and rabbit proteins (O'hUigin et al., 1990).

The primary translation product is the prepropeptide. It has been suggested that the bovine Apo A-1 prepropeptide like that of the human (Gordon et al., 1983) and the rat is post-translationally modified. In humans, intestinal proapo A-1 contains a hexapeptide extension which ends with Gln-Gln and this precursor was shown to be secreted by Hep G2, hepatocarcinoma cells in culture without proteolytic cleavage of the hexapeptide prosegment (Gordon et al., 1983). Thus it was suggested that Apo A-1 undergoes additional proteolytic processing before it is integrated into plasma HDL (Gordon et al., 1983). In the
cow, cleavage may occur after the conserved Gln-Gln dipeptide to give a mature Apo A-1 protein with an N-terminal aspartate (O'hUigin et al., 1990). The conversion of proApo A-1 to mature Apo A-1 is known to occur extracellularly by an enzyme present in plasma. Edelstein et al. (1988) also showed that this enzyme produced by a hepatocarcinoma cell line (Hep G2) secretes both Apo A-1 and the converting enzyme. The converting enzyme is activated by calcium, inhibited by EDTA, and converts proApo A-1 to Apo A-1 through a first order kinetic reaction (Edelstein et al., 1988). In another study, Chinese hamster ovary (CHO) cells transfected with human Apo A-1 secreted Apo A-1. Furthermore, 90% of the secreted Apo A-1 was the processed mature protein, and a portion of the secreted protein was associated with lipid (Mallory et al., 1987). Thus processing of Apo A-1 seems to take place prior to its secretion.

Role of Apo A-1

Apo A-1 is the major protein constituent of high density lipoprotein and it mediates the binding of HDL to cells. HDL is the major source of circulating cholesterol in bovine species (Sparrow et al., 1992). Pate and Condon (1989) showed that both LDL and HDL could be used as a source of cholesterol for steroidogenesis by bovine luteal cells, and both LDL and HDL enhance luteal progesterone synthesis in culture. In addition to its ability to solubilize and transport lipids, Apo A-1 is also a potent activator of lecithin-cholesteryl acyltransferase, the enzyme that catalyses formation of cholesterol esters from
cholesterol (Soutar et al., 1975). It has also been shown that HDL, as opposed to LDL, causes an increase in the release of placental lactogen by human placental explants (Handwerger et al., 1987), and from monolayer of trophoblast cells (Sane et al., 1988). Apo A-1 was implicated for the HDL-mediated stimulation of placental lactogen release. Wu et al. (1988) showed that HDL stimulates placental lactogen release by stimulating production of cAMP. Thus cAMP is a second messenger in HDL-mediated release of hPL, and HDL may carry out other functions in steroidogenic cells of the ovary by stimulating adenylate cyclase activity and cAMP production.

**Regulation of Apo A-1 Synthesis by Steroid Hormones**

There are reports to indicate that estrogen may regulate synthesis of Apo A-1 by the liver. Archer et al. (1986) reported that treatment of human hepatoma cell line (HepG2) with estradiol-17β, causes an increase in nuclear estrogen binding sites, and a parallel increase in the expression of Apo A-1 mRNA and rate of accumulation of the protein. The increase in mRNA levels accounted for 85-90% of the observed increase in rate of accumulation of secreted protein (Archer et al., 1986). A study with ovariectomized baboons showed that baboons treated with estradiol and progesterone had the highest serum concentrations of Apo A-1, followed by those treated with estrogen alone, and lowest in the progesterone-treated animals and the untreated controls. Baboons treated with progesterone alone had similar levels of serum Apo A-1 similar to
those of untreated controls (Kushwaha et al., 1990). Apo A-1 levels were significantly upregulated by estradiol and progesterone compared to untreated controls (Kushwaha et al., 1990).

Thyroid hormones also regulate expression of Apo A-1 mRNA. Apo A-1 gene is stimulated by triiodothyronine (T3) and has been shown to contain a thyroid hormone response element which is critical for the T3-induction of Apo A-1 mRNA and activity of Apo A-1 promoter (Romney et al., 1992). These observations were supported by reports from Chan et al. (1993) that the Apo A-1 gene contains a cis-regulatory element which acts on an adjacent site to increase promoter activity. HNF-4, a new member of the thyroid/steroid hormone receptor superfamily, was shown to interact with the cis element to enhance activity of the rat Apo A-1 promoter (Chan et al., 1993).

Effects of Nutrition on Apo A-1 Synthesis

Dietary carbohydrates or fatty acids regulate Apo A-1 gene expression by altering either gene transcription or mRNA stability. Synthesis and secretion of Apo A-1 is reduced in hepatocytes from rats fed fish oil (low source of cholesterol), but the diet did not affect levels of Apo A-1 mRNA (Ribeiro et al., 1992). Availability of cholesterol has been shown to enhance synthesis of Apo A-1 by human hepatoma cells (Craig et al., 1988). Similarly, Go et al. (1988) showed that synthesis of hepatic and intestinal Apo A-1 increases while levels of Apo A-1 mRNA decrease, following chronic fat and cholesterol feeding.
However, hepatic and intestinal synthesis of Apo A-1 is higher in African green monkeys than in Cynomolgus monkeys fed the same level of cholesterol (Sorci-Thomas et al., 1988). Thus other factors independent of dietary cholesterol intake may also regulate hepatic and intestinal Apo A-1 synthesis.

**Apolipoprotein E**

**Biochemical Characterization**

Apolipoprotein E (Apo E), sometimes referred to as arginine-rich protein, is a component of very low density lipoprotein (VLDL), HDL and LDL. Apolipoprotein E has been shown to have a molecular weight ranging between 33 and 39 kDa on SDS-PAGE (Shelburne and Quarfordt, 1974). Apolipoprotein E gene is 3597 nucleotides in length and contains four exons and three introns (Paik et al., 1985), and a similar gene structure is shared by other apolipoproteins. The primary translation product is a pre-Apo E protein with an 18-residue signal peptide that is cleaved cotranslationally (Zannis et al., 1984), and the mature protein is secreted.

The primary structure of Apo E ranges in length between 279 and 310 amino acid residues among different species. Apolipoprotein E sequence in the cow comprises 294 amino acid residues, and the most conserved region is between residues 28-61 (Yang et al., 1991). The receptor binding region (residues 130-158) is rich in basic amino acids and is conserved across species,
except for point substitutions in the dog (arginine substituted for lysine at 157) and cow (proline substituted for arginine at 145) (Weisgraber, 1994). Apolipoprotein E has been shown to exhibit heterogeneity in molecular weight and charge which have been attributed to genetic variation and posttranslational glycosylation with sialic acid. Sialo-Apo E isoforms comprise 42% of intracellular Apo E, 81.1% newly-secreted Apo E, and 24% plasma Apo E (Zannis et al., 1984). Thus sialation may be required for the secretion of Apo E or glycosylated Apo E is preferentially secreted. There has been evidence to suggest that Apo E is glycosylated by O-glycosidic linkage (Zannis et al., 1984).

Liver is the major source of Apo E. However, Apo E and its mRNA is produced by most organs and by several cell types within the organs including astrocytes, smooth muscle cells and macrophages (Mahley, 1988).

**Effects of FSH, LH, cAMP, and Phorbol Ester on Apo E Synthesis**

Apo E is synthesized by the rat ovary and represents 0.15% of the total protein synthesized in the ovary (Driscoll and Getz, 1984). Secretion of newly-synthesized Apo E by granulosa cells in culture is stimulated by FSH in a dose- and time-dependent manner, and the effects of FSH are mediated through cAMP (Driscoll et al., 1985). Results from that study also suggest that Apo E is secreted as part of a lipid-protein complex. As the granulosa cells differentiate in culture, they lose their responsiveness to FSH and cAMP (Driscoll et al., 1985). Polacek et al. (1992) showed that Apo E mRNA is localized
predominantly in theca cells of rat ovarian follicle, and mRNA levels increase following treatment of cells with hCG (Polacek et al., 1992).

In another study, Wyne et al. (1989a) demonstrated that BtcAMP and forskolin (an activator of adenylate cyclase and mediator of kinase A), and phorbol ester (mediator of kinase C) stimulate production of Apo E by granulosa cells in culture. BtcGMP (mediator of kinase G) did not stimulate secretion of Apo E (Wyne et al., 1989a). Kinases A and C had no effect on global protein synthesis in granulosa cells; incorporation of radiolabel into protein ranged between 10-15%, suggesting a specific stimulation of a subset of proteins including Apo E (Wyne et al., 1989a). In addition, cAMP, TPA and cholera toxin also stimulated expression of Apo E mRNA in rat granulosa cells. These agents stimulated accumulation of Apo E more than expression of its mRNA, indicating that kinases A and C may influence both the transcription of Apo E gene and the translational efficiency of Apo E mRNA. However, it is not yet clear if there is a crosstalk between the adenylate cyclase pathway (stimulated by cAMP) and the PKC pathway (stimulated by phorbol ester).

The stimulatory effect of cholera toxin and TPA on Apo E secretion is inhibited by cycloheximide and actinomycin D, suggesting that new proteins (such as transcriptional activator proteins AP-1 and AP-2) are required to mediate the stimulatory effects (Wyne et al., 1989a). The rat Apo E gene does not contain a cAMP regulatory region (CRE), but contains sequences with 75% homology to this region. Similarly, the consensus sequence for AP-1 (AP-1
responds to phorbol ester) is not present in the upstream region of the rat Apo E gene, but is found in the first intron. However, the consensus sequence for AP-2, which responds to both cAMP and phorbol ester, is present in the upstream region of Apo E gene (Wyne et al., 1989a).

Regulation of Apo E Synthesis by Cell Cholesterol

Cholesterol is the substrate for steroid hormone biosynthesis. Cholesterol can either be newly synthesized from acetate or is obtained by uptake of lipoproteins (Schreiber et al., 1980). Wyne et al. (1989b) demonstrated that inhibition of cholesterol synthesis from acetate with mevinolin, an inhibitor of HMG-CoA reductase, causes a decline in cholera-stimulated Apo E synthesis and expression of Apo E mRNA by rat granulosa cells. However, an inhibitor of the cytochrome P450 side chain cleavage enzyme had no effect on Apo E synthesis (Wyne et al., 1989b). Human and rat Apo E gene has been shown to possess the consensus sequence of a sterol regulatory element in their 5' region. Prack et al. (1991) also reported that depletion of adrenal gland cholesterol content decreases Apo E mRNA levels. Thus cholesterol together with stimulators of kinases A and C are required to regulate Apo E production.

Regulation of Apo E Secretion by Cytokines

Macrophage Apo E secretion decreases with macrophage activation (Zuckerman and O'Neal, 1994). This effect is mediated by macrophage
activating factors such as lipopolysaccharide (LPS) and granulocyte-macrophage colony stimulating factor (GM-CSF). The LPS-mediated reduction in Apo E secretion is inhibited by monoclonal antibody to murine tumor necrosis factor (TNF) (Zuckerman and O'Neal, 1994).

Role of Apo E in Ovarian Function

The role of Apo E in the ovary has not yet been identified. One possibility is that since Apo E mediates binding of lipoproteins to their receptors, Apo E could function to provide cholesterol for membrane and steroid hormone biosynthesis. Apo E may also function in a paracrine fashion to distribute lipid between ovarian cells and perhaps between compartments of the ovary. It has been demonstrated that HDL containing Apo E, as opposed to HDL containing no Apo E, induces rat ovarian theca cells to produce progesterone rather than androgen (Dyer et al., 1988).

Manganese Superoxide Dismutase

Biochemical Characterization

Superoxide dismutases play critical roles in protecting cells from oxidative damage by reactive oxygen species. Manganese superoxide dismutase (Mn SOD) is one of three (the others are Cu SOD and Zn SOD) enzymes that catalyze the dismutation of superoxide radicals to hydrogen peroxide and
Manganese SOD is localized in the mitochondrial matrix (Fridovich, 1974) and is not a secretory protein, while Cu SOD and Zn SOD are secretory proteins (Rueda et al., 1994). Manganese SOD has a molecular weight of 20 kDa. Two mRNAs transcripts of 4.0 kb and 1.0 kb encode for human Mn SOD (Melendez and Baglioni, 1993). However, three Mn SOD mRNA transcripts (1.5, 1.9, and 3.7 kb) have been observed in the bovine CL (Rueda et al., 1995; Ndikum-Moffor et al. 1995 - unpublished data. The mRNA transcripts are from the same gene, have identical coding regions, but differ in length of their 3' untranslated region (3' UTR) because of polyadenylation (Church, 1990). The 4-kb mRNA is expressed at a faster rate than the 1-kb mRNA, but the 4-kb transcript has a shorter half-life (2-4 h in different cells) than the 1-kb transcript (10-12 h) in both intact cells and a cell-free system (Melendez and Baglioni, 1993). The different half-lives indicate a post-transcriptional regulation of Mn SOD mRNA, and the instability of the 4-kb transcript has been attributed to the presence of AU-rich sequences in the 3' UTR (Melendez and Baglioni, 1993). Manganese SOD activities are low under normal physiological conditions, but may increase during differentiation and in response to oxidants and cytokines.

Regulation of Manganese SOD Production by Oxidative Stress

Reactive oxygen species are generated in all cells in vivo, and the toxicity of oxygen has been shown to be directly related to the production of oxygen-
dependent free radicals. Results from a study with yeast indicated that electron transport is a major source of superoxide anion \textit{in vivo} (Guidot et al., 1993). Oxidative stress from the environment has also been shown to increase production of mitochondrial Mn SOD in plants (Bowler et al., 1991).

**Regulation of Manganese SOD Synthesis by Gonadotropins**

In the rat, Laloraya et al. (1988) demonstrated a sharp increase in rat ovarian SOD activity 30 min following an injection of LH, a decline 60 min post-injection, and no LH-induced SOD activity in rats injected with anti-LH serum. They also observed changes in ovarian SOD activity across the estrous cycle, with highest levels at proestrus. However, changes in SOD activity specific to Mn SOD across the cycle were not discussed (Laloraya et al., 1988). In another study, Sato et al. (1992) showed that rat ovarian Mn SOD activity decreases during a hCG-induced ovulation, to a minimum 12 h post-injection, while Mn SOD mRNA levels increase markedly with time to a maximum 12 h post-hCG treatment (Sato et al., 1992).

**Regulation of Manganese SOD Synthesis by Cytokines and Phorbol Ester**

Interleukin-1, TNF, and lipopolysaccharide dramatically increase Mn SOD mRNA levels in pulmonary epithelial cells (Visner et al., 1990). Similar observations were reported by White and Tsan (1994) who also showed that TNF and IL-1 enhance Mn SOD protein and enzyme activity.
Whitsett et al. (1992) showed that TNF-alpha and phorbol ester (TPA) increase steady state mRNA and rate of transcription of human Mn SOD in pulmonary adenocarcinoma cells. The time course and extent of increased manganese SOD gene transcription by TNF-alpha was distinct from that exhibited by phorbol ester (Whitsett et al., 1992).

**Role of Mn SOD in the Ovary**

The role of SOD in the ovary has not been defined but there are indications that it might be involved in ovulation (Laloraya et al., 1988; Sato et al., 1992) and the luteolytic process (Wu et al., 1992; Rueda et al., 1995). It has been hypothesized that reactive oxygen species produced during normal metabolism may be potential mediators of luteal regression. A comparison of Mn SOD (a scavenger of superoxide radicals) gene expression between a functional CL (day 21 of pregnancy) and a regressed CL (day 21 of the estrous cycle) of the cow indicated that Mn SOD mRNA levels are higher in the functional than the regressed CL (Rueda et al., 1995). A lower expression of Mn SOD mRNA in the regressed CL suggests that cells within the regressed CL are less capable of metabolizing the superoxide radical, which may damage the cells and disrupt luteal function.
**Tissue Inhibitors of Metalloproteinases: TIMP-1 and TIMP-2**

Tissue inhibitor of metalloproteinases are proteins which inhibit the activity of enzymes (matrix metalloproteinases) that degrade protein components of the extracellular matrix. Thus the expression of TIMPs is high in tissues undergoing remodelling or transformation. Apart from their protease-inhibitory activity, TIMP-1 (Hayakawa et al., 1992) and TIMP-2 (Stetler-Stevenson et al., 1992) have also been shown to stimulate growth of erythroid cells, gingival fibroblasts, and transformed human lung cells. Satoh et al. (1994) also demonstrated that TIMP-1 stimulates growth of bovine embryos in culture. A recent study by Boujrad et al. (1995) showed that TIMP-1 secreted by rat Sertoli cells stimulated steroidogenesis by rat Leydig cells. Activities of matrix metalloproteinases (collagenases, stromelysins, and gelatinases) may be controlled at various levels, one of which is by binding to specific inhibitors (TIMP). Thus a proper balance is required between the amount of inhibitors and the metalloproteinases to maintain tissue homeostasis or proper remodeling which occurs during many biological processes. To date, three members of the TIMP family have been identified, namely TIMP-1, TIMP-2, and TIMP-3. Comparison of deduced amino acid sequence of TIMP-2 showed that TIMP-1 and TIMP-2 share 37.6% identity of nucleotide and 65.6% similarity of amino acid at the protein level (Stetler-Stevenson et al., 1990), while human TIMP-3 shares 39 and 46% amino acid sequence identity with human TIMP-1 and TIMP-
2, respectively (Silbiger et al., 1994). The positions of all twelve cysteine residues and three out of four tryptophans are conserved between TIMP-1 and TIMP-2.

**Tissue Inhibitor of Metalloproteinases-1**

**Biochemical Characterization**

TIMP-1 is a secreted glycosylated protein with molecular weight ranging from 28-30 kDa. TIMP-1 is expressed in many different tissues and cell types including monocytes, fibroblasts and macrophages. Ovine TIMP-1 shares 95, 86, and 77% nucleotide sequence with that reported for bovine, human, and mouse TIMP-1, respectively (Smith et al., 1994). The amino acid sequence of TIMP-1 deduced from the bovine cDNA sequence shows that the mature protein contains 12 cysteine residues (conserved among many species) and 2 N-glycosylation sites (Freudenstein et al., 1990). The nucleotide sequence of ovine TIMP-1 also indicates the presence of 12 cysteines and 2 N-linked glycosylation sites (Smith et al., 1994). In addition, TIMP-1 contains a 23-amino acid signal peptide which contains a core of hydrophobic amino acids (Smith et al., 1994).

TIMP-1 binds to active collagenase, and to the latent form of the 92 kDa gelatinase, and proteoglycans. TIMP-1 binds collagenase with high affinity in a 1:1 molar ratio to form an inactive noncovalent enzyme-inhibitor complex.
Regulation of TIMP-1 Synthesis by Gonadotropins

Mann et al. (1991) showed that LH and phorbol ester (TPA) individually increased metalloproteinase inhibitor activity of granulosa cells in culture in a dose-dependent manner, and the effects were additive. The inhibitor activity (identified as TIMP-1) was also stimulated by Br-cAMP and forskolin, and its mRNA levels increased before ovulation (Mann et al., 1991). In another study Mann et al. (1993) showed that cycloheximide inhibits basal, LH- and TPA-stimulated TIMP-1 activity, while indomethacin (an inhibitor of prostaglandin synthesis) or an antiestrogen did not affect basal or LH-induced rat granulosa cell inhibitory (TIMP-1) activity. Reich et al. (1991) also reported a lack of effect of eicosanoid on ovarian expression of TIMP-1. Rat granulosa cell TIMP-1 mRNA is also increased by LH and hCG, but the induced mRNA expression is not affected by cycloheximide (Mann et al., 1993). Thus de novo protein synthesis is required for LH- and TPA-induced increase in granulosa cell TIMP-1 activity but protein synthesis is not necessary for stimulation of TIMP-1 mRNA expression.

Luteal synthesis of TIMP-1 has been reported in the ewe (Smith et al., 1993; 1994), cow (Freudenstein et al., 1990; Ndikum-Moffor et al., 1995), rat (Mann et al., 1991), mouse (Edwards et al., 1992), and ferret (Huang et al., 1993). Results from some of these studies indicate that luteal synthesis of
TIMP-1 is triggered and stimulated by the surge in LH. Gonadotropins have also been shown to regulate expression of TIMP-1 mRNA in Sertoli cells. Treatment of prepubertal rat Sertoli cells with FSH and 8-bromo cAMP increases activity of TIMP-1, amount of TIMP-1 protein in conditioned-medium, and expression of TIMP-1 mRNA (Ulisse et al., 1994). Similar to observations in rat granulosa cells (Mann et al., 1991), de novo protein synthesis and RNA synthesis are required for both basal and TPA-, 8-bromo cAMP-, and FSH-stimulated TIMP-1 activity (Ulisse et al., 1994). The effects of phorbol esters on gene transcription occur through fos and jun containing AP-1 transactivating factors and the latter is induced by PKC-activating stimuli (Lee et al., 1987). On the other hand, cAMP enhances gene transcription by stimulating PKA which stimulates phosphorylation of cAMP response element binding proteins (CREB) (Sassone-Corsi et al., 1988; Merino et al., 1989).

The mechanism(s) through which TIMP-1 synthesis is stimulated has not been fully characterized. The murine TIMP-1 gene contains cis-acting regulatory elements upstream of the major transcription start site and also contains an AP-1 binding site within the cis-acting region (Edwards et al., 1992). The AP-1 functions as binding site for fos-jun and can stimulate transcription. Fos and Jun, like the CREB proteins are members of an extended basic region-leucine zipper (bZIP) superfamily of transcription factors. It has been shown that oligonucleotides containing a CREB sequence compete for binding of proteins to TIMP-1 AP-1 site. Thus the CREBP family may be involved in specific binding to
TIMP-1 AP-1 site (Edwards et al., 1992). Expression of TIMP-1 is stimulated by factors that increase intracellular cAMP. Thus TIMP-1 AP-1 site is the cis-acting regulatory element that mediates the cAMP-induced increase in TIMP-1 gene expression (Edwards et al., 1992). TIMP-1 enhancer element does not contain a classical CRE binding site but contains functional AP-1 sites, one of which can bind fos and jun heterodimers and other transacting factors including the CREB family (Edwards et al., 1992).

Effects of TPA on TIMP-1 synthesis are mediated through the PKC second messenger pathway since TPA-stimulated TIMP-1 activity is inhibited by an inhibitor of PKC (Staurosporine), and TIMP-1 activity is not stimulated by a non-PKC activating phorbol ester (Mackay et al., 1992).

**Regulation of TIMP-1 Synthesis by Steroid hormones**

Rajabi et al. (1991a) demonstrated that estradiol-17ß stimulates degradation of collagen type 1 in nonpregnant guinea pig cervix in vitro. In addition, the cervix has been shown to produce collagenase and its synthesis is stimulated by estrogens, interleukin-1ß, and PGE₂ (Rajabi et al., 1991b). Rajabi et al. (1991c) furthermore showed that activities of collagenase and collagenase inhibitor are greater in cervical tissue at the time of parturition than in tissues from nonpregnant animals. The marked increase in inhibitor activity observed at a time when collagenase activity is increased indicates the presence of a strong regulatory mechanism to control the extent of collagen degradation beyond the
level required for parturition (Rajabi et al., 1991c).

Progesterone stimulates TIMP-1 production by rabbit uterine cervical fibroblasts (Imada et al., 1994). Similar observations were reported by Sato et al. (1991) who showed that progesterone and estradiol-17β increases secretion of TIMP-1 by rabbit uterine cervical fibroblasts in culture and steady state TIMP-1 mRNA. However, observations by Rajabi et al. (1991c) showed that estradiol causes a decrease in tissue collagenase activity.

Retinoic acid has been shown to enhance secretion of TIMP-1 by human fibroblasts in vitro by increasing de novo synthesis of TIMP-1 (Clark et al., 1987). Retinoic acid also increased TIMP-1 mRNA levels compared to nontreated controls. Glucocorticoid treatment had no effect on TIMP-1 secretion (Clark et al., 1987).

**Regulation of TIMP-1 Synthesis by Cytokines and Growth Factors**

Synthesis of TIMP-1 and collagenase by human fibroblasts is stimulated by phorbol ester and IL-1 (Murphy et al., 1985). Similar effects of IL-1 were reported by Rajabi et al. (1991c) for cervical tissue of guinea pig. TIMP-1 activity and expression of its mRNA have been shown to increase in a variety of normal and tumor cell lines following treatment with IL-1 and tumor necrosis factor (TNF) (Mackay et al., 1992). Transforming growth factor-β (TGF-β) has also been implicated as a regulator of TIMP-1 synthesis. One of the mechanisms proposed for the control of normal trophoblast proliferation and
invasiveness by TGF-β is via induction of TIMP-1 mRNA expression. This regulatory mechanism is absent in malignant trophoblast cells (Graham et al., 1994).

Tissue Inhibitor of Metalloproteinases-2

Biochemical Characterization

Tissue inhibitor of metalloproteinases-2, the second member of the family of metalloproteinase inhibitors, binds and inactivates all matrix metalloproteinases but in contrast to TIMP-1 which binds the 92-kDa gelatinase, TIMP-2 binds the 72-kDa gelatinase. TIMP-2 has a molecular weight of 20-21 kDa and in contrast to TIMP-1, TIMP-2 is not glycosylated. In addition to its protease-inhibiting activity, TIMP-2 also possesses erythroid potentiating activity (Stetler-Stevenson et al., 1992), and has been shown to stimulate proliferation of skin fibroblast cells by stimulating cAMP and activating cAMP-dependent adenylate cyclase (Corcoran and Stetler-Stevenson, 1995).

Like TIMP-1, TIMP-2 contains 12 conserved cysteine and three tryptophan residues. The rat TIMP-2 gene encodes a 220 amino acid -long pro-TIMP-2 protein containing a 26-residue hydrophobic leader sequence, and a mature 194 amino acid protein (Santoro et al., 1994). TIMP-2 is expressed in a variety of cells and tissues, and it possesses two mRNA transcripts with approximate sizes of 3.5 and 1.0 kb (De Clerk et al., 1994).

There is evidence to suggest that although TIMP-1 and TIMP-2 possess
similar physiological properties, expression of their activity and mRNA seem to be differentially regulated in mouse reproductive tissues (Waterhouse et al., 1993). In contrast to the stimulating effects of phorbol ester on murine TIMP-1 mRNA, phorbol ester does not affect expression of TIMP-2 mRNA (De Clerk et al., 1994). In another study Waterhouse et al. (1993) reported a differential expression in TIMP-1 mRNA and TIMP-2 mRNA in the ovary of mice during gestation; TIMP-1 mRNA is low while TIMP-2 mRNA shows a marginal increase. Leco et al. (1992) also reported that while TIMP-1 mRNA is highly serum-inducible in normal murine fibroblasts, expression of TIMP-2 mRNA is mainly constitutive and is insensitive to transformation while expression of TIMP-1 mRNA is variable (Leco et al., 1992).

**Regulation of TIMP-2 Synthesis by Gonadotropins**

Like TIMP-1, TIMP-2 activity, TIMP-2 protein and mRNA levels in rat Sertoli cells are stimulated by FSH through a cAMP-dependent pathway (Ulisse et al., 1994).

**Regulation of TIMP-2 Synthesis by Steroid Hormones**

Production of TIMP-2 in culture by rabbit uterine cervical fibroblasts increases after treatment with physiological concentrations of progesterone (Imada et al., 1994). TIMP-2 mRNA is expressed constitutively in rat hepatocytes and its expression is up-regulated following incubation of
hepatocytes with dexamethasone and prostaglandin E$_2$ (Roeb et al., 1995).

**Regulation of TIMP-2 Synthesis by Cytokines and Growth Factors**

Transforming growth factor-β has been shown to down-regulate both mRNA transcripts of TIMP-2 in contrast to its stimulatory effect on TIMP-1 mRNA expression (Stetler-Stevenson et al., 1990). Mackay et al. (1992) also reported that TIMP-2 activities are refractory to TPA, IL-1 and TNF-α, in contrast to the marked stimulation of TIMP-1 activities by all three agents in a variety of human cell lines. A similar lack of stimulatory effect of TPA on TIMP-2 activity is also observed in rat Sertoli cells in culture (Ulisse et al., 1994).
Table 1-1. Summary of Factors Regulating Synthesis of Proteins.

<table>
<thead>
<tr>
<th>Regulation/Function</th>
<th>Apo A-1</th>
<th>Apo E</th>
<th>Mn SOD</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene Expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>E₂, T3,</td>
<td>FSH, LH, cAMP, hCG</td>
<td>LH, hCG, TPA, IL-1, TNF, LPS</td>
<td>FSH, cAMP P₄</td>
<td>FSH, cAMP P₄, PGE2, TGF-β</td>
</tr>
<tr>
<td></td>
<td>HNF-4,</td>
<td>CT, TPA, Cholesterol</td>
<td>TPA, IL-1, TNF, LPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Translation</strong></td>
<td>Dietary Cholesterol</td>
<td>cAMP, CT, TPA, TNF, LPS, GM-CSF</td>
<td>TNF, IL-1</td>
<td>IL-1, TNF, Vit A, P₄, E₂</td>
<td>FSH, cAMP</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Cholesterol, E₂</td>
<td>Cholesterol metabolism, membrane and steroid biosynthesis</td>
<td>Prevents oxidative stress</td>
<td>Tissue remodelling, cell growth, Steroid biosynthesis</td>
<td>Tissue remodelling, cell growth</td>
</tr>
</tbody>
</table>
CHAPTER 3
PROTEINS SYNTHESIZED AND RELEASED IN CULTURE BY THE BOVINE CORPUS LUTEUM: THE ESTROUS CYCLE AND PREGNANCY

Introduction

Apart from its traditional role in progesterone synthesis and maintenance of pregnancy, the corpus luteum synthesizes and secretes a number of proteins during the estrous cycle and pregnancy. The corpus luteum of the cow and ewe synthesizes the peptides oxytocin and neurophysin during the estrous cycle and stores these proteins in secretory granules of the large luteal cells (Fields et al., 1986; 1992) and ewe (Fields et al., 1986; Theodosis et al., 1986). The synthesis and secretion of proteins vary with the physiological status of the animal. For example, the number of oxytocin-containing secretory granules in the cow increases from metestrus to diestrus, and then declines prior to luteolysis (Fields et al., 1992), whereas in pregnancy the population of granules was undetectable on day 45, then increased to a peak between days 180-210 (Fields et al., 1985). However, the corpus luteum of mid-pregnancy (after day 45) in the cow contains neither the mRNA for oxytocin (Ivell et al., 1985) nor do the secretory granules contain oxytocin (Fields et al., 1992).

Additional proteins identified as secreted by the corpus luteum include the
tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2) in the rat (Parmer et al., 1992), sheep (Smith and Moor, 1991), Smith et al., 1995; Smith et al., 1993; 1994), cattle (Freudenstein et al., 1990; Juengel et al., 1994), pig (Smith et al., 1994)) and ferret (Huang et al., 1993), relaxin in humans and non-ruminants (Sherwood, 1994), inhibin in sheep (Tsonis et al., 1988; Rodgers et al., 1989; Smith et al., 1991), and insulin-like growth factor-1 (Einspanier et al., 1990), basic fibroblast growth factor (Stirling et al., 1991) and angiogenic factors (Redmer et al., 1988; Grazul-Bilska et al., 1992) in cattle. In addition to its role in maintenance of pregnancy, the corpus luteum of pregnancy in the cow appears to be necessary for normal parturition since removal during the third trimester resulted in increased rates of dystocia, retained fetal membranes (Estergreen, 1967), and greater death loss of calves (Tanabe, 1966). The synthesis and secretion of proteins by the bovine CL of pregnancy may play a role in setting the stage for parturition. The ferret corpus luteum was shown to secrete proteins on days 5-11 of pregnancy, with molecular masses of 16 to 185 kDa (Huang et al., 1993). Although no qualitative difference in protein secretion was observed across days of pregnancy studied, a 32 kDa protein that cross-reacted weakly with a polyclonal antibody to human TIMP was the most abundantly secreted protein (Huang et al., 1993).

The objectives of this study were to examine for proteins synthesized de novo by the bovine CL, identify and characterize the newly-synthesized proteins, and determine quantitative differences in their synthesis and release during the
estrous cycle and pregnancy.

**Materials and Methods**

**Materials**

Acrylamide was purchased from ICN Biomedicals Inc. (Cleveland, OH), bis-acrylamide and agarose from Bio-Rad Laboratories, N,N,N,N-tetramethylethylenediamine (TEMED) from Fisher Scientific (Fair Lawn, NJ), and ampholines from Pharmacia (Piscataway, NJ). Other electrophoretic reagents were obtained from Bio-Rad Laboratories (Richmond, CA). L-4,5-^H-leucine (specific activity 164 Ci/mmol) and D-[6-^H]glucosamine (specific activity 20Ci/mmol) were purchased from Amersham (Arlington Heights, IL), and $^{35}$S-methionine (specific activity 1028 Ci/mmol) was purchased from ICN Biomedicals Inc. Polyvinylidene fluoride (PVDF) was obtained from Millipore Corporation (Bedford, MA). Tissue culture media including amino acids, vitamins, insulin and antibiotic-antimycotic mixture, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

**Collection of Luteal Tissue**

Forty-eight Angus and Hereford crossbred beef cows were used for the study. All procedures in which animals were used were approved by the Animal Care and Use Committee of the University of Florida. Estrus (day 0) was
defined as that day when a cow would stand to be mounted by a bull. Cows randomly assigned to the pregnancy group were artificially inseminated at observed estrus, whereas cows assigned to the cycle group were not bred. Day 17 of pregnancy was confirmed by the presence of an embryo in flushings from the uterus. Later stages of pregnancy were estimated by measurement of crown-rump length of the fetus (Winters et al., 1942). Reproductive tracts were obtained from cows within 5 min after exsanguination at the University of Florida abattoir. The ovary containing the corpus luteum was collected aseptically from cows on days 3 (n = 4 cows), 7 (n = 3), 11 (n = 4), 14 (n = 5), 17 (n = 3), and 19 (n = 3) of the estrous cycle, and from cows of early pregnancy (day 17, n = 5), and the first (day 88, n = 5), second (day 170, n = 7), and third (greater than day 240, n = 9) trimester of pregnancy. Ovaries were immediately transferred to a sterile Petri dish (100 x 15 mm) containing pre-warmed Eagle's Minimum Essential Medium (MEM), and the corpus luteum dissected from the ovarian stroma and weighed.

**Culture Medium**

Medium was prepared as previously described (Basha et al., 1980) from Eagle's MEM deficient in leucine, lysine, methionine and sodium bicarbonate. One liter of stock incomplete MEM was prepared with the addition of glucose (3 g/l), methionine (1.5 mg/l), leucine (5.2 mg/l) and lysine-HCl (72.5 mg/l) to achieve 4.0, 0.1, 0.1, and 1.0 times, respectively, their usual concentrations in
MEM. Sodium bicarbonate (2.2 g/l), non-essential amino acids (1 %, v/v), vitamins (1%, v/v) and insulin (200 IU/l) were added and pH adjusted to 7.1-7.3. The medium was filter-sterilized (0.22 μm) (Corning Inc, Corning, NY) and stored at 4 C. For ^3H-leucine labelled cultures, methionine (1.5 mg/100 ml) and antibiotic-antimycotic (ABAM) mixture (1%, v/v) were added to the stock incomplete MEM to obtain leucine-deficient incomplete modified MEM. Similarly, for ^35S-methionine labelled cultures, methionine-deficient medium was prepared by adding leucine (5.2 mg/100 ml) and ABAM to the stock incomplete MEM. Incomplete MEM was used for ^3H-glucosamine labelled cultures.

**Time Course Studies of Incorporation of Radiolabel**

CL from three pregnant cows (two on day 170, and one on day 88) were used in the time course studies. Slices (0.5 mm in thickness) of luteal tissue were prepared with a Stadie-Rigg's tissue slicer (Thomas Scientific, Swedesboro, NJ). Explants were washed three times, each time with 15 ml incomplete MEM, to reduce serum proteins in the medium during incubation. After washing, luteal tissue (500 mg/dish) from the same corpus luteum was placed in four Petri dishes each containing 15 ml of leucine-deficient incomplete MEM without radiolabel. The dishes were pre-incubated at 37 C on a rocker platform for 2 h in an atmosphere of 50% N2:47.5% O2:2.5% CO2 (v/v/v). After pre-incubation, the medium was discarded and replaced with 15 ml leucine-deficient incomplete MEM containing 50 μCi of ^3H-leucine (160 Ci/mmol). Each
of the four Petri dishes of luteal tissue from each CL was then incubated as described for 6, 18, 24, and 30 h, respectively (ie n = 3 for each time point). At the end of each incubation, the luteal-conditioned medium (LCM) was separated from tissue by centrifugation at 2000 x g for 20 min at room temperature.

Medium was dialyzed immediately for 24 h at 4 C using Spectra/por 3 membrane (molecular weight cut off = 3500; Spectrum Medical Industries Inc., Houston, TX) against two changes (24 h each) of 4 l Tris-HCl buffer (10 mM, pH 8.2), and then against deionized water (two changes, 24 h each). Following dialysis, percent incorporation of radiolabel in the dialyzed LCM was determined for each incubation time. Percent incorporation was calculated as: post-dialysis radioactivity (dpm) divided by pre-incubation radioactivity (dpm) x 100%.

**Culture and Radiolabelling of Luteal Tissue**

The 24 h incubation was determined as the optimal time of incubation following incubations at 6, 18, 24, and 30 h as described. For each cow at least two Petri dishes of luteal slices (500 mg tissue/dish) were incubated as described in the above protocol for 24 h. Following incubation and dialysis, the total volume of dialyzed LCM (retentate) for each Petri dish was measured and adjusted to 15 ml with deionized water. Retentates were stored at -20 C until further analysis. Luteal slices (500 mg) were also incubated with ³H-glucosamine (50 μCi/15 ml, n = 1 cow, day 240 pregnant) or ³⁵S-methionine (50 μCi/15 ml, n = 1 cow, day 240 pregnant) to determine if newly-synthesized and
released proteins were glycosylated and/or contained methionine.

**TCA precipitation**

Following LCM dialysis, samples of dialyzed medium (retentate) for each corpus luteum were analyzed to determine the amount of $^3$H-leucine incorporated into trichloroacetic acid (TCA)-precipitable protein using a modification of method described by Mans and Novelli (1961). Briefly, a 50 µl aliquot of LCM retentate was spotted onto a 2.54 cm x 2.54 cm Whatman 3MM filter paper that was pre-soaked in 20% (w/v) TCA. Each square was air-dried, soaked in 20% and 5% TCA for 10 and 20 min, respectively, and subsequently washed twice, for 15 min each, in 95% ethanol. Squares were allowed to air-dry completely, placed in scintillation cocktail and counted for radioactivity. TCA-precipitable protein (dpm) was measured in duplicate for each LCM retentate sample, and values were expressed as least squares means (LSM) for each group.

**Light and Electron Microscopy**

To assess the effects of the 24 h-incubation on tissue morphology, luteal tissues collected before and after 24 h of culture with radiolabel were processed for evaluation by electron microscopy (Fields et al., 1992). Briefly, the central part of each CL was dissected into 1-3 mm cubes and fixed in 1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Cubes were
postfixed in 1% (v/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min, embedded individually in Spurr's medium, and sectioned (0.1 μm). Sections were observed and photographed using a Philips electron microscope (Model 301, Philips Electronic Instruments, Mahwah, NJ). For evaluation by light microscopy, luteal tissue was fixed in Bouins, embedded in paraffin, and sections were cut and stained with haematoxylin and eosin (Sheehan and Hrapchak, 1980).

**Two-Dimensional-SDS- Polyacrylamide Gel Electrophoresis**

**First-dimension: Isoelectric Focusing.** Proteins, synthesized and released into the medium of explant culture, were separated according to their isoelectric points (pI) by isoelectric focusing (IEF) as previously described (Laemmli, 1970). Frozen retentates were lyophilized and reconstituted in IEF gel sample buffer [9 M urea, 2% (v/v) NP-40, and 2% (v/v) ampholine (pH 3.5-10)]. Each sample (100,000 cpm) was loaded and the proteins separated in the IEF tube gel (4% (w/v) T, 5.4% (w/w) C_bis) by electrophoresis at 400 V for 20 h.

**Second-dimension: SDS-PAGE.** Following first dimension electrophoresis, the tube gels (100,000 cpm per gel) were equilibrated in gel-equilibration buffer [0.0625 M Tris, 5% (w/v) sodium dodecylsulfate (SDS), 10% (v/v) glycerol], for 15 min before they were loaded onto a slab SDS-polyacrylamide gel (14 cm x 16 cm x 0.15 cm; stacking gel 4% (w/v) T, 2.7% (w/w) C_bis; separating gel 10% (w/v) T, 2.7% (w/w) C_bis). Proteins in the IEF gel were separated on a slab gel by
electrophoresis in tank buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) at 13 mA/slab gel until the dye front reached the end of the gel (O'Farrel, 1975). Following electrophoresis, gels were stained with 0.1% (w/v) Coomassie blue, destained in destaining solution [50% (v/v) ethanol, 10% (v/v) acetic acid] and soaked in deionized water for 30 min. Gels were then treated with 1 M sodium salicylate for 30 min and dried on a slab gel dryer (Model SE 1150, Hoefer Scientific Instruments, San Francisco, CA), followed by exposure to x-ray film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY) for 4 weeks. Intensities of the radioactive spots on fluorographs were determined by densitometric scanning (E-C Apparatus Corporation, St. Petersburg, FL). The scanner was standardized to detect intensities between the lightest (background) and the darkest spot on fluorographs. The area under the curve for each spot scanned was measured using the trace mode of an electronic planimeter (Model 1250, Numonics Corporation, Lansdale, PA). Area measurements in cm² represent relative amounts of each newly-synthesized protein.

**Protein Blotting and Amino Acid Sequencing**

Following detection of newly synthesized proteins, proteins on wet 2-D gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation) by electroblotting at a constant voltage (20 V) in transfer buffer (2-[N-Morpholino]ethanesulfonic acid, 10 mM MES, pH 6, Sigma Chemical Co.) at 4°C for 16 h, according to method of Towbin et al. (1979). Membranes were
stained in 0.1% (w/v) Coomassie blue in 50% methanol for 5 min, destained in a solution of 50% ethanol and 10% acetic acid, rinsed extensively in distilled water, and then air dried. Proteins on membranes were subjected to N-terminal amino acid sequence analysis (Protein Sequencer Model 470 A/B, Applied Biosystems, Foster City, CA) by the Protein Chemistry Core Laboratory of the Interdisciplinary Center for Biotechnological Research (ICBR) at the University of Florida. A search of protein, RNA, and DNA data banks was conducted using the National Center for Biotechnology Information Database Search program (Devereux et al., 1984).

Progesterone Assay

Trunk blood samples collected into heparinized vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, NJ) from cows at time of slaughter were processed, and all plasma samples were analyzed for progesterone by RIA in a single assay, using the progesterone Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA). One hundred microliter (100 μl) plasma was assayed per tube. The kit originally designed for human serum was validated for use with cow plasma. Progesterone standards were prepared from a stock solution (5 μg progesterone/ml benzene) by diluting with ovariectomized cow plasma. A quantitative linear recovery of progesterone was obtained with two replicates of 0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 pg progesterone/100 μl ovariectomized cow plasma [Y = 1.595 + 0.864X; Y = amount of
progesterone measured (pg/ml) and $X =$ amount of progesterone added; $R = .99$. The detection limit of the assay, determined as two standard deviations above the zero dose level, was 9.5 pg/ml. The intra-assay coefficient of variation for the one assay was 6.5%. The slope (Mean ± SEM) of the standard curve using the progesterone standards provided in the kit (0, 10, 50, 200, 1000, 2000, 4000 pg/100 μl) was $-0.68 ± 0.13$ and the estimated doses at 20, 50, and 80% binding were 10.03, 1.31, and 0.18 ng/ml, respectively.

**Statistical Analysis**

Data for percent incorporation, radiolabeled TCA precipitable protein, CL weights and plasma progesterone were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analytical System (SAS, 1988). Data from densitometric scanning were subjected to analysis of variance to determine differences in protein secretion. The statistical model had day and reproductive status as the main effects with residuals as the error term. Values in the text are least square means (LSM) ± standard error of the mean (SEM). Orthogonal contrasts were performed to determine differences among days of the cycle and stages of pregnancy.
Results

Histology of Luteal Tissue

Results of microscopic examination of luteal tissue before and after 24 h-incubation showed there was no degeneration of the tissue as a result of incubation; there was no evidence of presence of collagen fibers or loss of secretory granules (Fig. 3-1). There was presence of secretory granules and intact mitochondria.

CL Weight and Plasma Progesterone

As expected, weight of the corpus luteum was different \((P < 0.004)\) across days of the estrous cycle, but not across stages of pregnancy (Fig. 3-2). Corpora lutea weights were lowest \((P < 0.002)\) on day 3 when compared to the other days of the estrous cycle examined. Weight of the corpus luteum was lower \((P < 0.03)\) on day 11 than 14. Plasma progesterone had a similar pattern as the corpora lutea weight, with concentrations varying \((P < 0.006)\) across days of the estrous cycle, but not across stages of pregnancy (Fig. 3-2). Plasma progesterone was lower \((P < 0.03)\) on day 3 when compared to the rest of the estrous cycle.
Figure 3-1. Electron micrograph of luteal cells.

A) Top panel: Luteal cell of tissue prior to incubation (control). Note secretory granules (arrow), nucleus at the bottom, and intact mitochondria. x 9,000; #50627.

B) Middle panel: Luteal cell of tissue post-24 h incubation without radiolabel (control). Note mature secretory granules (arrow), and nascent secretory granules associated with the Golgi apparatus, and intact mitochondria. x 11,400; #50633.

C) Bottom panel: Luteal cell of tissue post-24 h incubation with 50 μCi $^{3}$H-leucine. Note secretory granules (arrow) and nucleus at the bottom. Intact mitochondria is not different from that of the controls. x 11,400; 50630.
Figure 3-2. Least square means ± SEM of plasma concentrations of progesterone (ng/ml) and weight (g) of the corpus luteum across days 3, 7, 11, 14, 17, and 19 of the estrous cycle and days 17, 88, 180, and >240 of pregnancy. Progesterone concentrations and CL weight increased from day 3 to day 14 of the estrous cycle and then declined to day 19, but these did not vary across pregnancy.
Plasma progesterone was lower ($P < 0.03$) during early luteal (day 7) than mid-luteal phase (days 11 and 14) of the estrous cycle. Similarly, plasma $P_4$ was lower ($P < 0.09$) on day 11 than day 14, and day 19 was lower than day 17 ($P < 0.05$). However, there were no differences between day 17 of the estrous cycle and day 17 of pregnancy for plasma progesterone ($4.79 \pm 1.1$ vs $5.36 \pm 1.3$, $P < 0.76$) and CL weight ($4.66 \pm 0.7$ vs $4.83 \pm 0.6$, $P < 0.87$).

**Incorporation of Radiolabel into TCA-precipitable Protein**

Results of the time course study ($n = 3$ cows) showed that the 24 h-incubation time was optimum for incorporation of radiolabel when compared with the 6, 18, and 30 h incubation time (Fig. 3-3), thus 24 h-incubation time was used in this study. There was no lag time, the rate of incorporation increased sharply between 18 h and 24 h of incubation, and did not change or declined between 24 and 30 h. Analysis of all LCM retentates following dialysis indicated that there was no difference in the amount of radioactivity associated with TCA-precipitable proteins released in culture across days of the estrous cycle or stages of pregnancy. The amount (LSM ± SEM) of TCA-precipitable radioactivity was not different ($P < 0.23$) between day 17 ($3104.6 \pm 500.5$ dpm) of the estrous cycle and day 17 ($2217.6 \pm 433.5$ dpm) of pregnancy. Percent
incorporation of radiolabel across the estrous cycle ranged between 2.4 ± 0.8% (day 11) and 6.2 ± 0.9% (day 19) and approached significance ($P < 0.09$). The percent incorporation ranged between 2.0 ± 0.6% and 3.4 ± 0.6% during pregnancy, did not differ across pregnancy, and was not different ($P < 0.35$) between day 17 of the estrous cycle and day 17 of pregnancy.

Figure 3-3. Time course studies of incorporation of radiolabel (n = 3 cows).
Figure 3-4. Percent incorporation of radiolabel into newly-synthesized proteins.
Luteal Protein Synthesis and N-terminal Amino Acid Micro Sequencing

The bovine CL synthesized and released a number of different proteins during the estrous cycle and pregnancy with molecular masses ranging from 12 to 200 kDa. Following 2D-SDS-PAGE and fluorography, eleven discrete radiolabeled proteins were selected for further study. For convenience, before the amino acid sequence information was available, each of the eleven major proteins was assigned a number; proteins 1 (35 kDa, pl 5.5), 2 (30 kDa, pl 5.5), 3 (29 kDa, pl 5.5), 4 (27 kDa, pl 5.5), 5 (70 kDa, pl 5.0), 6 (58 kDa, pl 6.0), 7 (44 kDa, pl 5.0), 8 (30 kDa, pl 8.0), 9 (20 kDa, pl 8.0), 10 (22 kDa, pl 8.0), and 11 (27 kDa, pl 6.0) (Fig. 3-5, 3-6). Five of these proteins were identified from their N-terminal amino acid sequence. Between 18 and 31 N-terminal amino acid residues for proteins 1, 8, 9, 10 and 11 were determined following separation by 2D-SDS-PAGE and transfer to PVDF membranes (Table 3-1). Protein 1, composed of four protein spots, and proteins 8 and 11, each composed of two protein spots were individually subjected to N-terminal micro-sequence analysis. A search of protein data banks matched these N-terminal amino acid sequences to those of bovine apolipoprotein E (Apo E; all four protein spots associated with protein 1),
Figure 3-5. Representative fluorographs of proteins synthesized de novo in explant culture and released into the medium by CL on different days of the estrous cycle. Proteins 1, 2, 3, and 4 were observed only on day 3. Other identified proteins 5-10 were found on all other days (7-19) and protein 11 was found on days 11-19.
Figure 3-6. Representative fluorographs of proteins synthesized de novo in explant culture and released into the medium by CL on different days of pregnancy. Proteins 5-11 were found on all days of pregnancy (17, 88, 180, and > 240) examined.
Table 3-1. Comparison of N-terminal amino acid sequences of proteins 1, 8, 9, 10 and 11 in luteal-conditioned medium to sequences in the protein data banks*.

<table>
<thead>
<tr>
<th>Protein, Apo E (35 kDa, pI 5.5)</th>
<th>Lab Sequence:</th>
<th>Bovine Apo E:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp Met Glu Gly Glu Leu Gly Pro Glu Glu Pro Leu – – Gln Gln Pro Arg Gly Lys</td>
<td>Asp Met Glu Gly Glu Leu Gly Pro Glu Glu Pro Leu Thr Thr Gln Gln Pro Arg Gly Lys</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein 8, TIMP-1 (30 kDa, pI 8)</th>
<th>Lab Sequence:</th>
<th>Bovine TIMP-1:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>– Thr – Val Pro Pro His Pro Gln Thr Ala Phe – Asn Ser Asp Val Val Ile Arg</td>
<td>Cys Thr Cys Val Pro Pro His Pro Gln Thr Ala Phe Cys Asn Ser Asp Val Val Ile Arg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein 9, TIMP-2 (20 kDa, pI 8)</th>
<th>Lab Sequence:</th>
<th>Bovine TIMP-2:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>– – – Pro Val His Pro Gln Gln Ala Phe – Asn Ala Asp Ile Val Ile Arg Ala Lys Ala Val Asn</td>
<td>Cys Ser Cys Ser Pro Val His Pro Gln Gln Ala Phe Cys Asn Ala Asp Ile Val Ile Arg Ala Lys Ala Val Asn</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein 10, Mn SOD (22 kDa, pI 8)</th>
<th>Lab Sequence:</th>
<th>Human Mn SOD:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>– Ser Leu Pro Asp Leu Pro Tyr Asn Tyr Gly Ala Leu Glu Pro His Asn Ala Glu Ile Met Gln Leu</td>
<td>Lys His Ser Leu Pro Asp Leu Pro Tyr Asn Tyr Gly Ala Leu Glu Pro His Asn Ala Glu Ile Met Gln Leu</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein 11, Apo A-1 (27 kDa, pI 6)</th>
<th>Lab Sequence:</th>
<th>Bovine Apo A-1:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg His Phe Trp Gin Gin Asp Asp Pro Gln Ser Ser Trp Asp Arg Val Lys Asp Phe Ala Thr Val Try Val Glu Ala Ile Lys Asp Ser Gly</td>
<td>Arg His Phe Trp Gin Gin Asp Asp Pro Gln Ser Ser Trp Asp Arg Val Lys Asp Phe Ala Thr Val Try Val Glu Ala Ile Lys Asp Ser Gly</td>
</tr>
</tbody>
</table>

*Dashes (−) indicate undetermined residues. The method used in the sequence analysis does not identify underivatized cysteines (Cys), while serines (Ser) and threonines (Thr) are low responders. The first two cycles in the run for TIMP-2 and Mn SOD were missed due to machine error.
bovine TIMP-1 (both protein spots associated with protein 8), bovine TIMP-2 (protein 9), human manganese superoxide dismutase (Mn SOD; protein 10), and bovine apolipoprotein A-1 (Apo A-1; both protein spots associated with protein 11). Although a few amino acid residues within four of the five sequences were unidentified, all identified residues matched those of the corresponding known sequence. Sequences for other determined proteins were not obtained by N-terminal sequence analysis due to insufficient quantities of protein for transfer.

**Protein Synthesis and Release During the Estrous Cycle**

Analysis of densitometric data of fluorographs indicated qualitative and quantitative differences in protein synthesis and release across days of the estrous cycle. Proteins 1 (Apo E), 2, 3, and 4 were observed only on day 3 (Fig. 3-7). Synthesis of protein 11 (Apo A-1) was not observed until day 11, and levels of release did not vary \( P < 0.35 \) across the remaining days of the estrous cycle although synthesis and release of Apo A-1 appeared to be higher on day 19 than on day 17 \( P < 0.11 \). The remaining proteins 5, 6, 7, 8 (TIMP-1), 9 (TIMP-2), and 10 (manganese SOD) were observed on all days except day 3. The relative amount of protein 8 (TIMP-1) synthesized and released into medium did not differ across days of the cycle, but appeared to increase on day 19 (day 17 < day 19; \( P < 0.11 \)). Synthesis and release of TIMP-2 and manganese SOD analyzed as a combined protein due to overlap on the gel did not vary \( P < 0.20 \) across the estrous cycle. However, orthogonal contrasts showed that synthesis
and release of TIMP-2 and SOD were higher during late rather than mid-luteal phase of the estrous cycle (days 17, 19 > days 7, 11, 14, $P < 0.01$). Although there was no overall difference in the release of protein 6 across days of the estrous cycle ($P < 0.13$), while protein 7 did vary ($P < 0.004$) across the estrous cycle. Orthogonal contrast analysis showed that protein 6 was greater ($P < 0.01$) during the late luteal phase (days 17 and 19) when compared with the mid-luteal phase (days 7, 11 and 14) of the estrous cycle, and greater on day 17 than 19 ($P < 0.08$). A greater amount of protein 7 was synthesized during late rather than the early and mid-luteal phases (days 17 and 19 > 7, 11 and 14, $P < 0.009$), and was also greater on day 19 compared to 17 ($P < 0.002$). The amount of protein 5 synthesized and released was at the minimum detectable limit on all days considered, and did not change during the estrous cycle.

**Luteal Protein Synthesis and Release During Pregnancy**

Corpora lutea of pregnancy synthesized and released proteins similar to those released by CL of the estrous cycle, except for Apo E and proteins 2-4 which were not observed during pregnancy (Fig. 3-7). No differences were observed in the synthesis of Apo A-1 ($P < 0.42$), protein 6 ($P < 0.21$), protein 7 ($P < 0.32$), TIMP-1 ($P < 0.29$), and TIMP-2 and manganese SOD ($P < 0.39$), released across pregnancy. However, orthogonal contrasts showed that TIMP-1 synthesis tended to be lower on day 180 when compared with day 240 or greater of gestation ($P < 0.18$). Similarly, synthesis of protein 6 was greater on day $>240$ than day 170 ($P < 0.16$), and synthesis of protein 7 on day 17 of
Figure 3-7. Densitometric analysis of fluorographs of newly-synthesized proteins in luteal-conditioned medium during the estrous cycle and pregnancy. Values are least square means ± standard error of the mean. Statistical differences were reported for protein 7 across the cycle, and for Apo A-1 across pregnancy. Orthogonal contrasts revealed the following differences: Apo A-1: day 17 < day 19 ($P < 0.11$), TIMP-1: day 17 < day 19 ($P < 0.11$), TIMP-2: days 7, 11 and 14 < days 17 and 19 ($P < 0.01$), protein 6: days 7, 11, 14 < days 17 and 19 ($P < 0.01$), and day 17 cycle > day 19 ($P < 0.08$), protein 7: days 7, 11 and 14 < days 17 and 19 ($P < 0.009$), and day 17 < day 19 ($P < 0.002$).
gestation tended to be higher than on the other days of pregnancy examined ($P < 0.14$). Synthesis of radiolabeled protein 5 was at the minimum detectable limit on all days considered and did not change during pregnancy. Synthesis and release in culture of all proteins, except the TIMP-2/SOD complex, did not differ quantitatively when comparing day 17 of the estrous cycle and day 17 of pregnancy. Qualitatively, the same proteins were observed on both days.

**Radiolabelled Culture with $^3$H-glucosamine and $^{35}$S-methionine**

Radioactivity patterns, detected by fluorography of gels of LCM retentate of a late pregnant cow, varied with the radiolabel used in the culture. The number of radioactive spots on fluorographs was greater following incubation with $^3$H-leucine than for incubations with $^3$H-glucosamine and $^{35}$S-methionine. For example, only protein 6 and TIMP-1 were detected after incubations with $^3$H-glucosamine suggesting that they are glycosylated, and all the other proteins 5, 7, 9-11, were not glycosylated (Fig. 3-8). Protein 7 was the only protein not detected after incubations with $^{35}$S-methionine indicating little or no presence of methionine in this protein (Fig. 3-9). Thus, $^3$H-leucine was used in all subsequent incubations. Proteins 1-4 were not evaluated since they were not synthesized by the corpus luteum of during pregnancy.
Figure 3-8. Representative fluorographs of proteins synthesized de novo in explant culture and released into medium on day 240 of pregnancy.
A) Left panel: Radiolabelled culture with $^3$H-leucine. Proteins 5-11 were observed.
B) Right panel: Radiolabelled culture with $^3$H-glucosamine. Proteins 6 and 8 were observed. Proteins 5, 7, 9-11 were not observed.
Figure 3-9. Representative fluorographs of proteins synthesized de novo in explant culture and released into medium on day 240 of pregnancy.

A) Left panel: Radiolabelled culture with $^3$H-leucine. Proteins 5-11 were observed.

B) Right panel: Radiolabelled culture with $^{35}$S-methionine. Proteins 5, 6, 8-10 were observed. Proteins 7 and 11 were not observed.
Discussion

In the present study, the bovine corpus luteum was shown by explant culture techniques and gel electrophoresis and fluorography to synthesize and release proteins differentially across the estrous cycle and pregnancy. Major proteins had molecular masses of 20 to 70 kDa and pI's from 5.0 to 8.0. N-terminal amino acid micro sequence analysis identified five of these proteins with significant sequence identity to bovine Apo E, bovine Apo A-1, bovine TIMP-1, bovine TIMP-2, and human manganese SOD. The amounts of protein synthesized and released by the corpus luteum in explant culture was variable during the estrous cycle but and pregnancy. Bovine luteal Apo E was synthesized and released only on day 3 of the estrous cycle.

This is the first report of the synthesis and release of Apo E by the bovine CL. Bovine Apo E was synthesized and release as isoforms, suggesting that newly-synthesized and released bovine luteal Apo E may be post-translationally modified. Apo E is synthesized as a preprotein and post-translationally modified with the addition carbohydrate chains such as sialic acid (Zannis et al., 1984). Higher concentrations of the glycosylated form of Apo E in newly- secreted protein than in intracellular forms of Apo E have been described by Zannis et al. (1984). In addition, newly-secreted Apo E has a higher sialic acid content than plasma Apo E (81 ± 11% vs 24 ± 6%), and these results would suggest the possibility of variable posttranslational modifications which could lead to several
different isoforms and Mr forms.

Apo E is a ligand for the low density lipoprotein receptor and may be involved in regulating movement of cholesterol into the corpus luteum. Synthesis and release of Apo E only on day 3 suggests it may regulate cholesterol availability for membrane and/or steroid synthesis during early luteal development. Rat granulosa cells have been shown to produce Apo E (Driscoll et al., 1985; Wyne et al., 1989), and it has been proposed that secretion of Apo E early in the differentiation of the follicle to a corpus luteum may facilitate the shift from androgen production, required for follicular estrogen to progesterone synthesis during luteinization. However there was no evidence for the synthesis of Apo E by luteal cells has been reported (Polacek et al., 1992). A model for autocrine and paracrine involvement of Apo E in peripheral nerve regeneration following injury has been proposed (Mahley, 1988). A parallel model for the ovary could involve a wound healing effect of Apo E, due to ovulation and massive reorganization of the ovulated follicle into a corpus hemorrhagicum. Three other proteins with similar isoelectric points but lower molecular weights have been characterized and were observed only on day 3 of the cycle. To date these proteins have not been identified.

Synthesis and release of Apo A-1 was not observed on days 3 and 7 of the estrous cycle, but was observed on all other days of the estrous cycle and pregnancy examined. This is the first report of synthesis of Apo A-1 by the corpus luteum of any species. Apo A-1 is synthesized primarily in the liver and
small intestine but has also been shown to be produced by peripheral tissues and secreted in culture (Blue et al., 1982). The amino acid sequence of the 27 kDa protein has a 100% identity with bovine Apo A-1 (O'hUigin et al., 1990) and has therefore been identified as Apo A-1 in the present study. Apo A-1 is a major protein in the high density lipoprotein complex (HDL) which is the major source of circulating cholesterol in the cow (Sparrow et al., 1992). Thus Apo A-1 may function to regulate the availability of cholesterol to steroidogenic cells within the corpus luteum, ovary, and placenta. In the case of luteal synthesis of Apo A-1, a paracrine role is most likely given the demand for cholesterol by the corpus luteum. There is evidence that Apo A-1 levels are higher in serum from a female with the presence of a corpus luteum than in the serum of an anestrous animal (Oikawa and Katoh, 1995). However, the contribution of synthesized and secreted luteal apolipoproteins to circulating levels, if any, is unknown at this time.

Secretion of TIMP-1 was first detected on day 7 of the estrous cycle and was higher during late luteal phase of the cycle, and did not vary in general during pregnancy, although levels tended to be higher during late pregnancy. Studies in sheep have shown that the ovine corpus luteum secretes a number of proteins on day 10 of the cycle, and TIMP-1 was the most abundant (Smith et al., 1991; 1993). In the present study, the 30 kDa protein released into medium has a 100% amino acid sequence similarity to bovine TIMP-1 (Freudenstein et al., 1990). While the role of TIMP in the corpus luteum has not been clarified,
TIMPs in general are specific inhibitors of enzymes that degrade protein components of the extracellular matrix (Werb, 1989). Controlled remodelling of the extracellular matrix has been shown to take place during ovulation (Le Maire, 1989), uterine implantation (Lala et al., 1990) and gestation (Brenner et al., 1989). Therefore, TIMP-1 may be involved in controlling remodeling of luteal tissue during its rapid phase of development and during regression.

Additionally, TIMP-1 and TIMP-2 possess erythroid-potentiating activity (Stetller-Stevenson et al., 1992), while TIMP-1, isolated from bovine granulosa cell-conditioned medium, has been shown to promote the growth of bovine embryos in vitro (Satoh et al., 1994). A recent study (Boujrad et al., 1995) has shown that TIMP-1, secreted by rat Sertoli cells, stimulates steroidogenesis by rat Leydig cells, in a dose-dependent manner. TIMP-1 may have a multiplicity of roles in the CL, including those described here but, more importantly, role(s) which may still be undefined. However, the increasing TIMP-1 secretion by a regressing corpus luteum (plasma progesterone < 0.5 ng/ml) on day 19 was unexpected.

In the present study, TIMP-2 and manganese SOD co-migrated in 2-D-SDS-PAGE gels. The resultant protein spot was subjected to N-terminal amino acid sequence analysis which indicated the presence of manganese SOD, a primary sequence, and TIMP-2, as the secondary sequence within the protein spot. Synthesis and release of the TIMP-2 and manganese SOD complex varied across the estrous cycle and was greater during later stages of the cycle.
However, synthesis of this complex did not differ across pregnancy. Greater synthesis of TIMP-2 and expression of its mRNA by the ovine corpus luteum was shown earlier rather than later in the cycle (Smith et al., 1995), while in the mouse ovary, TIMP-2 mRNA was shown to increase with advancing gestation (Waterhouse et al., 1993). These differences may reflect species specificity. The physiological role of TIMP-2 in the corpus luteum is not clear. Like TIMP-1, TIMP-2 may be involved in luteal development, tissue remodelling and regression.

Manganese SOD is a mitochondrial protein (Church, 1990). There have been no reports to suggest that manganese SOD is a secretory protein. In the present study, synthesis of manganese SOD was undetectable on day 3, but was observed through the rest of the cycle. The bovine large luteal cell has been shown to be densely packed with mitochondria which swell during the cycle and take on dense inclusions as early as day 14 (Fields et al., 1992). This will suggest that luteal manganese SOD synthesized and released in explant culture could have resulted from an involutive process in which mitochondrial membranes become compromised.

Another reason for the increase in manganese SOD during the involutive phase of the estrous cycle could be an attempt by the corpus luteum to protect elf from deleterious oxygen radicals. Luteal manganese SOD may protect the corpus luteum from oxidative damage by converting reactive superoxide anions to hydrogen peroxide and oxygen (Laloraya et al., 1988; Hesla et al., 1992).
However, others have shown that hydrogen peroxide inhibits hormone-sensitive steroidogenesis in rat luteal cells by the uncoupling of occupied LH receptors from adenylate cyclase (Musicki et al., 1994), and inhibiting synthesis of proteins involved in the intracellular movement of cholesterol to mitochondrial cytochrome P450 side chain cleavage enzyme (Musicki et al., 1994). A recent study has shown that a functional bovine corpus luteum (day 21 of pregnancy) expresses higher levels of manganese SOD than a regressed corpus luteum on day 21 of the estrous cycle (Rueda et al., 1995). The authors suggested that a decline in the synthesis of manganese SOD and other enzymes that prevent oxidative stress may contribute to the luteolytic process (Rueda et al., 1995). In the present study, the combined amount of newly-synthesized TIMP-2 and manganese SOD present in the medium were higher on day 17 of the estrous cycle than on day 17 of pregnancy.

Interestingly, protein synthesis and secretion by the corpus luteum was observed as late as day 19 of the estrous cycle, despite the decline in plasma progesterone and regression of the corpus luteum at this time (Fields et al., 1992). Seemingly, the corpus luteum maintains the capability to synthesize proteins independently of steroidogenesis suggesting differential regulation of these two functions. These results are consistent with those previously described by Juengel et al. (1994) who also observed protein synthesis and secretion by bovine corpora lutea during luteolysis. In their study, two of the proteins produced, TIMP-1 and TIMP-2, were secreted in amounts observed to
change during the PGF$_{2a}$-induced luteolysis (Juengel et al., 1994). In the present study, the bovine corpus luteum synthesized and released a number proteins during involution and regression. The roles these proteins play in the luteolytic process may be significant.

In conclusion, the bovine CL synthesized and released in culture at least eleven proteins, some of which were identified as Apo E, Apo A-1, TIMP-1 and TIMP-2 and Mn SOD. These may act in an autocrine, paracrine, and/or endocrine manner to regulate luteal development, tissue remodelling, and steroidogenesis.
CHAPTER 4
EXPRESSION OF MESSENGER RNA OF APOLIPOPROTEINS E AND A-1 IN BOVINE LUTEAL TISSUE DURING THE ESTROUS CYCLE AND PREGNANCY

Introduction

An earlier study (chapter 3) showed that bovine luteal tissue synthesizes and releases apolipoprotein E (Apo E) and apolipoprotein A-1 (Apo A-1) in explant culture. The apolipoproteins shared 100% amino acid sequence identity with bovine Apo A-1 (O'hUigin et al., 1990) and with bovine Apo E (Weisgraber, 1994). The synthesis and release of Apo A-1 by the corpus luteum of any species, and of Apo E by the corpus luteum of the cow is novel.

Apo E is a constituent of very low density lipoproteins (VLDL), low density lipoprotein (LDL), chylomicrons, and certain subclasses of high density lipoprotein (HDL). Apo E mediates the binding of Apo E-containing lipoproteins to the LDL receptor (Mahley, 1988b). Apolipoprotein E is secreted by granulosa and theca cells of the rat (Wyne et al., 1989b), and has been shown to make up approximately 0.15% of the total proteins synthesized in the rat ovary (Driscoll et al., 1985). The theca cells have been shown to be the primary site of Apo E synthesis in the rat ovary (Wyne et al., 1989b). In a more recent study, Polacek et al. (1992) showed that expression of Apo E mRNA within ovarian follicles is
differentially localized and developmentally regulated. Apo E mRNA is primarily located in theca cells of immature preantral follicles. The level of Apo E mRNA expression was shown to increase following maturation of the follicle and following treatment with gonadotropins. Granulosa cells of the Graafian follicle show greater expression of Apo E mRNA than cells of the preantral follicle (Polacek et al., 1992).

Apo E mRNA expression in granulosa cells is stimulated by agents that stimulate cAMP production (Wyne et al., 1989a) including FSH and cholera toxin that stimulate granulosa cell Apo E secretion in a dose and time-dependent manner (Driscoll et al., 1985). Activation of protein kinases A and C, but not kinase G, independently stimulate synthesis and secretion of Apo E (Wyne et al., 1989a). Polacek et al. (1992) found no evidence of expression of Apo E mRNA in luteal tissue of the rat.

Apo A-1 is the major protein of HDL (Sparrow et al., 1992). It is synthesized mainly by the liver but also by other peripheral tissues (Blue et al., 1982; Ferrari et al., 1986). Shackelford and Lebherz (1983) have shown that synthesis of Apo A-1 by the chick liver, skeletal and smooth muscle, is developmentally regulated. It has also been reported that breast muscle of the chick directs more of its protein synthetic efforts to the production of Apo A-1 than does the liver, around the time of hatching (Ferrari et al., 1986). Our previous study showed that Apo A-1 is synthesized by the bovine corpus luteum during the estrous cycle and pregnancy, and the amount synthesized and
released did varied across days the estrous cycle and pregnancy examined (chapter 3).

The objectives of the present study were to determine the presence, and temporal expression of mRNA for Apo E and Apo A-1 in bovine luteal tissue across the days of the estrous cycle and pregnancy.

**Materials and Methods**

**Materials**

All electrophoresis reagents were of electrophoresis grade and were purchased from Fisher Scientific (Atlanta, GA). Biotrans nylon membrane was obtained from ICN Biomedicals (Costa Mesa, CA). The nick translation labeling kit was obtained from Amersham International plc (Buckinghamshire, England). $[^{32}P]$dCTP alpha was purchased from ICN Biomedicals. RNA molecular weight markers were from Gibco BRL (Life Technologies Inc., Gaithersburg, MD). XAR-5 film was obtained from Eastman Kodak (Rochester, NY). Prostaglandin F$_{2\alpha}$ was obtained from Upjohn (Kalamazoo, MI).

**Tissue Collection**

Angus and Hereford crossbred cows were used for the study. All procedures in which animals were used were approved by the Animal Care and Use Committee of the University of Florida. Estrus (day 0) was determined by observing cows twice daily for standing to be mounted by a bull rendered
incapable of mating. Cows were bred by artificial insemination at observed estrus. Day 17 pregnancy was confirmed by the presence of an embryo in flushings from the uterus. Later stages of pregnancy were estimated by measurement of crown-rump length of the calves (Winters et al., 1942).

Reproductive tracts were collected within 5 min after exsanguination from cows on days 2-3 (n = 4), 7 (n = 1), 16-17 (n = 4) and 20 (n = 2) of the estrous cycle, and on days 17 (n = 5), 90-120 (n = 2), 170-180 (n = 3) and > 215 (n = 5) of pregnancy. The corpus luteum was dissected from surrounding ovarian stroma, snap-frozen in liquid nitrogen, and then stored at -80°C until further analysis.

**Isolation of RNA**

Total cellular RNA was isolated from luteal tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Puissant and Houdebine, 1990). Fresh or frozen tissue (1 g) was homogenized on ice in a solution containing 10 ml 4 M cold guanidinium thiocyanate, 1 ml 2 M sodium acetate (pH 5.0) and 78 μl 2-mercaptoethanol, using a polytron tissue homogenizer (Tissumizer®, Tekmar Company, Cincinnati, OH). Total RNA was extracted from homogenate with a phenol-chloroform mixture (10 ml:2 ml) and centrifuged at 4000 x g for 15 min. The upper phase was treated with isopropanol and centrifuged to precipitate the RNA. The pellet was resuspended in 4 M lithium chloride, repelleted by centrifugation, and dissolved in RNA buffer (10 mM Tris, 1 mM EDTA, 0.5% (w/v) SDS, pH 7.5). RNA in solution was treated with
chloroform and the upper phase collected, treated with 2 M sodium acetate and isopropanol, and stored at -80 C to reprecipitate the RNA. Pure RNA pellet was retrieved after centrifugation at 4000 x g for 10 min and dissolved in sterile water. The concentration and purity (260 : 280 nm ratio) of the RNA were determined. Integrity of the RNA preparations was assessed by electrophoresis on a 1.2% (w/v in 1X TAE buffer) agarose gel. Total RNA samples were stored at -80 C until further analysis.

Preparation of Plasmid DNA

Plasmid DNA containing cDNA of Apo A1 (clone pAI-113) and Apo E (clone pE-301) from human liver (Breslow et al., 1982) was purchased from American Type Cell Culture (ATCC, Rockville, MD). Each plasmid DNA was reconstituted in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and spread on tet^R LB plates at 37 C overnight. Single bacteria clones were picked and added to 200 ml LB broth containing tetracycline (0.1 mg/ml), and then incubated at 37 C overnight with shaking. The plasmid DNA in the overnight culture was purified using the QIAGEN protocol (QIAGEN Inc., Chatsworth, CA), based on the optimized alkaline lysis method of Birnboim and Doly (1979). The incubation mixture was centrifuged at 5000 x g for 15 min at 4 C. The plasmid DNA in the pellet was lysed by suspending the pellet in an alkaline solution. The lysate was neutralized with acidic potassium acetate. The mixture was centrifuged to precipitate denatured proteins, chromosomal DNA and cellular
debris. The supernatant containing the plasmid DNA was then loaded on a resin (QIAGEN-tip column). The DNA was eluted from the column with elution buffer containing 1.25 M NaCl (pH 8.5). The eluted plasmid DNA was desalted and concentrated by isopropanol precipitation. The solution was centrifuged at 20,000 x g for 45 min at 4 C. The DNA pellet was washed with 70% ethanol and centrifuged again. The purified plasmid DNA pellet was lyophilized and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Yield was determined by measuring DNA concentration on a spectrophotometer at 260 nm, and the purity of the plasmid cDNA was assessed on a 1.2% agarose gel.

**Restriction Analysis and Isolation of Insert**

Plasmid preparations from the clones of Apo E and Apo A1 were digested with PstI at 37 C for 16 h. Plasmid digests were separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Fragments were transferred by electroblotting to DEAE cellulose membrane (Schleicher and Schuell, Keene, NH), and then extracted from membrane with a high salt buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8). Extracted inserts were reprecipitated with 3 M sodium acetate and 100% ethanol, then centrifuged at 12,000 x g to repellet. The DNA pellet was freeze dried and dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Size of purified inserts were checked on a 1.2% agarose gel by comparison to a series of RNA markers.

Digestion of the Apo E cDNA-containing plasmid with PstI yielded two
fragments of about 500 and 300 base pairs. Fragments were pooled before labeling by nick translation. Digestion of Apo A-1 cDNA-containing plasmid (pAl-113) with PstI yielded a single fragment of 600 base pairs.

**Northern Hybridization**

To determine the size of mRNA for Apo E and Apo A-1 in luteal tissue of different reproductive states, total RNA (30 μg) was denatured in denaturing buffer (24 mM HEPES, 6 mM sodium acetate, 1.2 mM EDTA, 50% (v/v) deionized formamide, 2.2 M formaldehyde) for 1 h on ice, followed by incubation at 65°C for 15 min. Denatured samples were separated by electrophoresis at 20 V for 16 h on a 1.5% agarose-formaldehyde gel in 1X running buffer (0.5 M NaH₂PO₄, 0.5 M Na₂HPO₄, pH 7). The gels were stained with ethidium bromide, and the presence of distinct bands of the 28 S and 18 S ribosomal RNA was used to assess the integrity of the RNA (Sambrook et al., 1989).

Fractionated RNA was transferred by capillary blotting to nylon membrane (Biotrans, ICN) using the TurboBlotter™ (Schleicher and Schuell) in 20X SSC (single-strength SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Filters were exposed to ultraviolet light for 90 sec to immobilize the RNA, then prehybridized for 2 h at 42 C in prehybridization buffer (5X Denhardt, 4X SSC, 0.5 M sodium phosphate pH 6.5, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) followed by hybridization at 42 C for 16 h in buffer (1X Denhardt, 4X SSC, 0.5 M sodium phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v)
formamide) containing $^{32}$P-cDNA probes (10$^6$ cpm/ml of hybridization buffer)
labeled by nick translation (Amersham International plc). Blots were washed
twice in double-strength SSC-0.1% SDS at 42 C for 15 min each, with shaking,
followed by two washes in 0.1-strength SSC-0.1% SDS at 42 C for 15 min each.

**Autoradiography**

Blots were exposed to x-ray film (Kodak XAR, Eastman Kodak) and an
intensifying screen at -80 C to detect hybridization signals. For hybridization
with a different probe, blots were washed with hot (90 C) 10% SDS for 1 h. In
this study, blots were rehybridized with a β-actin cDNA probe at 42 C to assess
the amount and integrity of total RNA loaded in each lane.

**Dot Blot Hybridization**

Total RNA (10 μg) was denatured in denaturation buffer (20 mM Tris, pH
7.0, 50% formamide, 6% formaldehyde), followed by incubation at 65 C for 5
min. Denatured samples were immobilized on membrane (Biotrans, ICN) using
a microsample filtration unit (Schleicher and Schuell). Filters were exposed to
ultraviolet light for 90 sec to crosslink the RNA to the membrane, then
prehybridized in buffer (5X Denhardt, 4X SSC, 0.5 M sodium phosphate pH 6.5,
0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) at 42 C for 2 h,
followed by hybridization in buffer (1X Denhardt, 4X SSC, 0.5 M sodium
phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide)
containing the $^{32}$P-cDNA ($10^6$ cpm/ ml of hybridization buffer) labeled by nick translation (Amersham International plc) at 42 C for 16 h. Blots were washed twice with 2X SSC, 0.1% SDS at 42 C for 15 min each on a shaker, followed by two washes with 0.1X SSC, 0.1% SDS at 42 C for 15 min each. Blots were exposed to x-ray film (Eastman Kodak) and/or phosphorimaging cassettes, and hybridization signals were quantified on a phosphorimager (ImageQuant and Phosphorimager, Molecular Dynamics Inc., CA). The relative expression of each mRNA to that of β-actin was used to calculate least square means.

Statistical Analysis

Data from dot blot analyses were analysed by Least Squares Analysis of Variance of the General Linear Models of the Statistical Analytical System (SAS, 1988). The statistical model had day and reproductive status as the main effects with residuals as error term. Values in the text are least square means (LSM) ± standard error of the mean (SEM). Orthogonal contrasts were used to determine differences among days of the estrous cycle and stages of pregnancy.
Results

Northern Blot Analysis of Apo E and Apo A-1 mRNA

Northern blot analysis revealed the presence of a single Apo E mRNA (1.0 kb) in bovine luteal tissue. Apolipoprotein E mRNA was expressed only during the early luteal phase (days 2-3). No hybridization was detected on the other days of the cycle or during pregnancy (Fig. 4-1). Apolipoprotein A-1 cDNA hybridized with a single mRNA transcript of approximately 1 kb in size (Fig. 4-2). Northern blots showed negligible expression of Apo A-1 mRNA on days 2-3 of the cycle. However, expression of Apo A-1 mRNA was detected on day 7, continued throughout the remainder of the estrous cycle and during pregnancy.

Dot Blot Analysis

In agreement with results obtained with northern blots, dot blot analysis showed expression of Apo E mRNA only on days 2-3 of the estrous cycle (Fig. 4-3). Although negligible in northern blots, dot blots revealed expression of Apo A-1 mRNA on days 2-3 (Fig. 4-4). Although expression of Apo A-1 mRNA was not different across days of the cycle, it did vary with stage of pregnancy ($P < 0.01$). Expression of Apo A-1 mRNA was at its lowest on days 90-120, and increased to significantly higher levels during the second half of pregnancy.
Figure 4-1. Northern blot analysis of apolipoprotein E mRNA. The same blot was probed with β-actin that served as a control for the loading and the integrity of the RNA. Expression of Apo E mRNA (1.0 kb) was observed only on days 2-3 of the estrous cycle, and was absent on the other days of the estrous cycle (7, 16, 17, and 20) and pregnancy (17 and 90) examined.
Figure 4-2. Northern blot analysis of apolipoprotein A-1 mRNA. The same blot was probed with β-actin that served as a control for the loading and the integrity of the RNA. Expression of Apo A-1 mRNA (1.0 kb) was not observed on days 2 and 3 of the estrous cycle, was expressed on day 7 and continued through the other days of the estrous cycle (7, 16, 17, and 20) examined. Apo A-1 mRNA was expressed on days 17, 90, 170, 180, and day 272 of pregnancy.
Figure 4-3. Dot blot analysis of Apo E mRNA. Ten µg total RNA isolated from CL during the estrous cycle (days 2-3, 16-17, and 20) and pregnancy (days 17, 90-120, 170-180, and > 215) was loaded per sample. RNA blots were hybridized with $^{32}$P-labelled Apo E cDNA. Expression of Apo E mRNA was observed only on days 2-3 of the estrous cycle, was not observed on the other days of the cycle (16-17, and 20) and pregnancy (17, 90, 170, and 272) examined.
(days 90-120 vs days 170-180 and 215 or greater; \( P < 0.002 \)), with no difference \((P < 0.47)\) in expression between days 170-180 and days 215 or greater of gestation (Fig. 4-5).

Figure 4-4. Dot blot analysis of Apo A-1 mRNA. Ten \( \mu \)g total RNA isolated from CL during the estrous cycle (days 2-3, 16-17, and 20) and pregnancy (days 17, 90-120, 170-180, and > 215) was loaded per sample. RNA blots were hybridized with \(^{32}\text{P}\)-labelled Apo A-1 cDNA. Expression of Apo A-1 mRNA was observed on all days of the estrous cycle (2-3, 16-17, and 20) and pregnancy (17, 90-120, 170-180, and > 215) examined.
Figure 4-5. Apo A-1 mRNA expression relative to β-actin is presented as LSMean ± SEM. Levels of Apo A-1 mRNA expression were similar across days of the estrous cycle, but were higher during the second compared with the first half of pregnancy.
Discussion

In addition to the presence of radiolabeled apolipoprotein E and apolipoprotein A-1 in luteal-conditioned medium following incubations with ³H-leucine (chapter 3), the presence of mRNA for apolipoproteins A-1 and E in bovine luteal tissue is further evidence for synthesis of these proteins by luteal tissue. This is the first report of expression of mRNA for apolipoproteins A-1 by the corpus luteum of any species, and for apolipoprotein E by the bovine corpus luteum. Nicosia et al. (1992) reported the presence of mRNA for apolipoprotein E in regressed corpus luteum of the rat. In the present study, bovine luteal tissue showed a single hybridization signal with the apolipoprotein E cDNA probe, with size of the Apo E mRNA transcript (1.0 kb) similar to that expressed in rat ovarian follicles (Polacek et al., 1992). Apolipoprotein E mRNA was expressed only during the early luteal phase (days 2-3). No expression of Apo E mRNA was observed on the other days of the estrous cycle and pregnancy examined.

Studies in the rat have shown that Apo E mRNA is expressed primarily by theca cells of the ovarian follicle. Levels of expression of Apo E mRNA have been shown to be lower in granulosa cells, and become undetectable with formation of the corpus luteum (Polacek et al., 1992). However, Nicosia et al. (1992) observed expression of Apo E mRNA in theca cells and regressed corpus luteum, but not in granulosa cells of the rat. In the present study, Apo E mRNA
was expressed only during early luteal phase (days 2-3) of the estrous cycle, the only period when the bovine corpus luteum has been observed to synthesize and release Apo E in explant culture (chapter 3).

Apolipoprotein E is a constituent of liver-synthesized very low density lipoprotein (VLDL) and of a subclass of high density lipoprotein (HDL) (Mahley, 1988). The significance of luteal synthesis Apo E, and its production only during the early luteal phase of the estrous cycle is not understood. We suggest that it may act in an autocrine and/or paracrine fashion to provide cholesterol for membrane biosynthesis and cell proliferation at a time when the corpus luteum is undergoing rapid reorganization and growth following the trauma of ovulation (Zheng et al., 1993). A similar role of apolipoprotein E in nerve regeneration following injury has been suggested (Mahley, 1988). Apolipoprotein E also functions as a heparin binding protein, thus may compete with other heparin binding growth factors (fibroblast growth factor -2, FGF-2), influencing luteal cell interaction with the extracellular matrix (Mahley, 1988). Zheng et al. (1993) reported the presence of FGF-2 in the bovine corpus luteum during the estrous cycle.

Immunohistochemical staining showed apolipoprotein E was restricted to the large steroidogenic luteal cells of days 3, 7, 11, and 14 of the estrous cycle. No staining was observed in the corpus luteum of pregnancy (F.M. Ndikum-Moffor, P.A. Fields, M.J. Fields - unpublished data). It appears that apolipoprotein E may be localized in secretory granules since immunostaining
was specifically localized in a region close to the nucleus, characteristically occupied by a cluster of secretory granules (Fields et al., 1992). Thus, even though synthesis of apolipoprotein E is restricted to the first days of the forming CL, it appears the protein is stored in the large luteal cell for release later in the estrous cycle. The temporal relationship of mRNA, \textit{de novo} synthesis, and immunohistochemical staining is in a manner very similar to that observed for bovine luteal oxytocin (Ivell et al., 1987; Fields et al., 1992). Since macrophages and monocytes have been shown to produce large quantities of Apo E (Driscoll et al., 1985), these cells may also contribute to luteal synthesis of Apo E.

Synthesis of apolipoprotein E and presence of its mRNA only during the early luteal phase indicate presence of a tightly controlled regulatory factor. A good candidate would be the gonadotropins and agents that stimulate cAMP and protein kinase A, which have been shown to increase the production of Apo E (Driscoll et al., 1985) and its mRNA (Polacek et al., 1992).

Dot blot analysis revealed the presence of Apo A-1 mRNA in bovine luteal tissue in which expression was similar across the estrous cycle, but differed across pregnancy. Level of expression was higher with advancing stages of pregnancy. A single hybridization signal (1.0 kb) was observed similar to that expressed by liver and other tissues (Ferrari et al., 1986). There is precedence for extrahepatic secretion of apolipoprotein A-1 in a variety of tissues including the yolk sac, gut, adrenal, kidney, human brain (Hopkins et al., 1986), pig brain (Guttler et al., 1990), liver, kidney, muscle, intestine, and testis.
of the rooster (Blue et al., 1982), baboon hepatocytes (White et al., 1994), and genital ridge of human embryos (Vorob'ev and Perevozchikov, 1992).

Apo A-1 is the major protein component of high density lipoprotein (HDL), the primary form of circulating cholesterol in the bovine species (Sparrow et al., 1992). The role of Apo A-1 in the CL can only be speculative at this point of our investigation. Pate and Condon (1989) showed that bovine luteal cells require lipoproteins for steroidogenesis. Although LDL is 3.5 times more potent than HDL, the latter is more important in vivo in the cow because of its higher concentrations in blood (Pate and Condon, 1989). It has also been shown that HDL is required in maintaining bovine luteal cells in culture, and that luteal cells utilize both LDL and HDL as a source of cholesterol for steroidogenesis (Pate and Condon, 1989). Luteal Apo A-1 may play an autocrine and/or paracrine role in the CL and ovary to provide cholesterol for steroidogenesis.

Prostaglandin F2α, the natural luteolysin in cattle has been shown to inhibit the lipoprotein-induced increase in progesterone production by bovine luteal cells (Pate and Condon, 1989), not by inhibiting cholesterol entry into the cells, but by inhibiting its utilization for steroidogenesis. In a recent study, Grusenmeyer and Pate (1992) reported that PGF2α does not affect lipoprotein-induced increase in cellular or mitochondrial cholesterol content but instead, has an effect after cholesterol transport to mitochondria, but prior to cholesterol side chain cleavage.
It has been suggested that Apo A-1 interacts with HDL to regulate placental biosynthesis of cholesterol and other events involved with onset of labor (Del Priore et al., 1991). The observed increase in luteal Apo A-1 mRNA as pregnancy progressed is congruent with a role for this protein during pregnancy. Serum concentrations of Apo A-1, HDL, and cholesterol in women have been shown to increase as pregnancy progressed, decline to normal levels during the periparturient period, and decline further with onset of labor (Del Priore et al., 1991). Apolipoprotein A-1 has also been shown to increase production of placental lactogen by human trophoblast cells by stimulating cAMP (Wu et al., 1988). An increase in luteal apolipoprotein A-1 during pregnancy could have a systemic effect on the placenta, although it is more likely that it is involved in an autocrine and/or paracrine role at the level of the ovary. The cell types responsible for luteal synthesis of apolipoprotein A-1 have not been identified, but studies to investigate that are underway. Identification of the cells may give additional insight into the function of luteal apolipoprotein A-1.

In conclusion, the bovine corpus luteum expressed mRNA for apolipoproteins E and A-1 during the estrous cycle and pregnancy. Although the role of these proteins in the corpus luteum is undefined, they may act in an autocrine and/or paracrine manner to regulate luteal development and/or steroidogenesis.
CHAPTER 5
EXPRESSION OF MESSENGER RNA OF TISSUE INHIBITOR OF METALLOPROTEINASES- 1 AND -2 IN BOVINE LUTEAL TISSUE DURING THE ESTROUS CYCLE AND PREGNANCY

Introduction

Luteal cells of domestic ruminants have the structural machinery required for the synthesis and secretion of proteins or peptide hormones. Tissue inhibitors of metalloproteinases (TIMP) are produced by different cells and tissues. In the cow (Freudenstein et al., 1990; Juengel et al., 1994), ewe (Smith et al., 1993; 1995), and pig (Tanaka et al., 1992), the corpus luteum (CL) has been shown to synthesize TIMP-1 and TIMP-2. In an earlier study (chapter 3), we showed that bovine luteal tissue synthesizes and releases tissue inhibitor of metalloproteinases-1 and 2 during the estrous cycle and pregnancy. TIMPs are inhibitors of enzymes that degrade the protein component of the extracellular matrix. Apart from their protease inhibitory activity, TIMP-1 (Hayakawa et al., 1992) and TIMP-2 (Stetler-Stevenson et al., 1992) have been shown to promote the growth of different types of cell. TIMP-1 enhances growth of bovine embryos in culture (Satoh et al., 1994). More recently, Boujrad et al. (1995) have shown that TIMP-1 of testicular origin stimulates the synthesis of pregnenolone by Leydig cells, and progesterone by Sertoli cells of the rat testis.
TIMP-1 is a 28-30 kDa glycosylated protein whereas TIMP-2 has a molecular mass of 20 kDa and is not glycosylated. In addition, TIMP-1 has a sequence homology with the granulocyte macrophage colony stimulating factor (GM-CSF) in the N-terminal region whereas TIMP-2 has no such sequence (Patarca and Haseltine, 1985). Waterhouse et al. (1993) have also reported that the temporal expression of TIMP-1 is different from that of TIMP-2 in reproductive tissues of the rat, suggesting that these proteins are independently regulated (Waterhouse et al., 1993).

Earlier reports in sheep (Smith et al., 1994) and cattle (Freudenstein et al., 1990) examined changes in expression of TIMP-1 and TIMP-2 mRNA during the estrous cycle. There is a paucity of information about the patterns of expression of mRNA for TIMP-1 and TIMP-2 during pregnancy. The objectives of this study were to determine expression of TIMP-1 and TIMP-2 in bovine luteal tissue, and differences in expression across the estrous cycle and pregnancy.

Materials and Methods

Materials

All electrophoresis reagents were of electrophoresis grade and were purchased from Fisher Scientific (Atlanta, GA). Biotrans nylon membrane was obtained from ICN Biomedicals (Costa Mesa, CA). The nick translation labeling
kit was obtained from Amersham International plc (Buckinghamshire, England). [32P]dCTP alpha was purchased from ICN Biomedicals (Costa Mesa, CA). RNA molecular weight markers were from Gibco BRL (Life Technologies Inc., Gaithersburg, MD). XAR-5 film was obtained from Eastman Kodak (Rochester, NY). Prostaglandin F2α was obtained from Upjohn (Kalamazoo, MI).

**Tissue Collection**

Angus and Hereford cross-bred cows were used for the study. All procedures in which animals were used were approved by the Animal Care and Use Committee of the University of Florida. Estrus was synchronized in cows by injection of 25 mg prostaglandin F2α (Lutalyse, Upjohn). Cows were observed for estrous behavior twice daily with assistance of a bull. Some cows were bred by artificial insemination at observed estrus (estrus = day 0). Day 17 pregnancy was confirmed by the presence of an embryo in flushings from the uterus. Later stages of pregnancy were estimated by measurement of crown-rump length of the calves (Winters et al., 1942). Reproductive tracts were collected within 5 min after exsanguination from cows on days 2-3 (n = 3), 7 (n = 1), 16-17 (n = 4) and 20 (n = 2) of the estrous cycle, and on days 17 (n = 5), 90-120 (n = 2), 170-180 (n = 3) and > 215 (n = 5) of pregnancy. The CL was dissected from surrounding ovarian stroma, snap-frozen in liquid nitrogen, and then stored at -80°C until further analysis.
**Isolation of RNA**

Total cellular RNA was isolated from luteal tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Puissant and Houdebine, 1990). Fresh or frozen tissue (1 g) was homogenized on ice in a solution containing 10 ml 4 M cold guanidinium thiocyanate, 1 ml 2 M sodium acetate (pH 5.0) and 78 μl 2-mercaptoethanol, using a polytron tissue homogenizer (Tissumizer®, Tekmar Company, Cincinnati, OH). Total RNA was extracted from homogenate with a phenol-chloroform mixture (10 ml:2 ml) and centrifuged at 4,000 x g for 15 min. The upper phase was treated with isopropanol and centrifuged to precipitate the RNA. The pellet was resuspended in 4 M lithium chloride, repelleted by centrifugation, and dissolved in RNA buffer (10 mM Tris, 1 mM EDTA, 0.5% (w/v) SDS, pH 7.5). The RNA in solution was treated with chloroform and the upper phase collected, treated with 2 M sodium acetate and isopropanol, and stored at -80°C to reprecipitate the RNA. Pure RNA pellet was retrieved after centrifugation at 4,000 x g for 10 min and dissolved in sterile water. The concentration and purity (260 : 280 nm ratio) of the RNA were determined. Integrity of the RNA preparations was assessed by electrophoresis on a 1.2% (w/v) in 1X TAE buffer (Tris, Acetic acid, EDTA) agarose gel. Total RNA samples were stored at -80°C until further analysis.
Preparation of Plasmid DNA

Plasmid DNAs containing cDNA of TIMP-1 (clone 6-2) and TIMP-2 (clone MMI) in agar stabs were a gift from Dr. Michael F. Smith of the University of Missouri, Columbia, MO (Smith et al., 1995). Each plasmid cDNA (100 µl) was suspended in 1 ml LB medium containing ampicillin (100 mg/l), and placed at 37 C overnight with shaking. The bacteria culture was grown on LB-Amp plates at 37 C overnight. A single bacterial colony was picked from the LB-Amp plate and suspended in 200 ml LB medium containing ampicillin at a final concentration of 100 µg/ml, and then incubated with shaking at 37 C overnight with shaking. The culture was centrifuged at 7,000 x g for 10 min at 4 C, and the plasmid DNA in the pellet was lysed by suspending the pellet in an alkaline mixture containing 5.8 ml ST buffer (25% sucrose, 50 mM Tris, pH 8.0), 1.95 ml lysozyme solution (10 mg/ml in ST) for 5 min. The mixture was centrifuged briefly, placed on ice for 5 min. Then 4.75 ml 0.2 M EDTA was added, followed by addition of 12.5 ml Triton Lysis buffer (1 ml 10% Triton X-100, 5 ml 1 M Tris (pH 8.0), 62.5 µl 0.1 M EDTA to 100 ml with sterile water). The mixture was centrifuged at 12,000 x g for 30 min to precipitate denatured proteins, chromosomal DNA and cellular debris. The supernatant containing the plasmid DNA was treated with 0.026X its volume of 8 M ammonium acetate. The DNA in the supernatant was extracted in a phenol-chloroform mixture and repelleted by centrifugation. The DNA pellet was lyophilized and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5).
Yield was determined by measuring DNA concentration on a spectrophotometer at 260 nm, and the purity of the plasmid cDNA was assessed on a 1.2% agarose gel.

**Restriction Analysis and Isolation of Insert**

Plasmid DNA of TIMP-1 and TIMP-2 were digested following a double digestion protocol. For TIMP-1, the digestion reaction was carried out with Xho I restriction enzyme for 16 h, followed by EcoR1 at 37 C for 16 h. For TIMP-2, the cleavage reaction was done with Bam H1 at 37 C for 16 h, followed by EcoR1 at same temperature for 16 h. Plasmid digests were treated with RNase for 30 min at 37 C, and separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. The fragments were transferred by electroblotting to DEAE cellulose membrane (Schleicher and Schuell, Keene, NH), and then extracted from membrane with a high salt buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8). The extracted inserts were reprecipitated with 3 M sodium acetate and 100% ethanol, then centrifuged at 12,000 x g to repellet. The DNA pellet was freeze dried and dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Sizes of purified inserts were checked on a 1.2% agarose gel by comparing with a series of RNA markers.
Northern Hybridization

Size of mRNA of TIMP-1 and TIMP-2 in luteal tissue was determined by northern blot analysis (Sambrook et al., 1989). Total RNA (30 μg) was denatured in buffer (24 mM HEPES, 6 mM sodium acetate, 1.2 mM EDTA, 50% (v/v) deionized formamide, 2.2 M formaldehyde) for 1 h on ice, followed by incubation at 65 C for 15 min. Denatured samples were separated by electrophoresis at 20 V for 16 h on a 1.5% agarose-formaldehyde gel in 1X running buffer (0.5 M NaH₂PO₄, 0.5 M Na₂HPO₄, pH 7). The gels were stained with ethidium bromide, and the presence of distinct bands of the 28s and 18s ribosomal RNA was used to assess the integrity of the RNA.

Fractionated RNA was transferred by rapid downward capillary blotting to nylon membrane (Biotrans, ICN Biochemicals) using the TurboBlotter™ (Schleicher and Schuell) in 20X SSC (single-strength SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Filters were exposed to ultraviolet light for 90 sec to immobilize the RNA, then prehybridized for 2 h at 42 C in 5X Denhardt (1X = 0.002% w/v ficoll, 0.02% w/v polyvinylpyrrolidone, 0.02% w/v BSA), 4X SSC, 0.5 M sodium phosphate pH 6.5, 0.1% w/v sodium dodecyl sulfate (SDS), yeast RNA (0.25 mg/ml), 50% (v/v) formamide). Filters were hybridized at 42 C for 16 h in buffer (1X Denhardt, 4X SSC, 0.5 M sodium phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) containing ³²P-TIMP-1 or ³²P-TIMP-2 cDNA (10⁶ cpm /ml hybridization buffer) labeled by nick translation.
Filters were washed twice in double-strength SSC-0.1% SDS at 42 °C for 15 min each, followed by two washes in 0.1-strength SSC-0.1% SDS at 42 °C for 15 min each.

**Autoradiography**

Filters were exposed to x-ray film (XAR, Eastman Kodak) and an intensifying screen for 4 days at -80 °C to detect hybridization signals. For hybridization with a different probe, blots were washed with hot (90 °C) 1% SDS for 1 h. Blots were rehybridized with a β-actin cDNA probe at 42 °C to assess the amount and integrity of total RNA loaded in each lane.

**Dot Blot Hybridization**

Total RNA (10 μg) was denatured in denaturation buffer (20 mM Tris, pH 7.0, 50% formamide, 6% formaldehyde), followed by incubation at 65 °C for 5 min. The denatured samples were immobilized on membrane (Biotrans, ICN) using a microsample filtration unit (Schleicher and Schuell). The filters were exposed to ultraviolet light for 90 sec to crosslink the RNA to the membrane, then prehybridized in buffer (5X Denhardt, 4X SSC, 0.5 M sodium phosphate pH 6.5, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) at 42 °C for 2 h, followed by hybridization in buffer (1X Denhardt, 4X SSC, 0.5 M sodium phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) containing the ^32P-cDNA TIMP-1 or TIMP-2 (ICN Biomedicals) labeled by nick
translation (Amersham International plc) at 42 C for 16 h. The blots were washed twice with 2X SSC, 0.1% SDS at 42 C for 15 min each time, followed by two washes with 0.1X SSC, 0.1% SDS at 42 C for 15 min each time. The blots were exposed to x-ray film (Eastman Kodak) and phosphorimaging cassettes, and hybridization signals were detected and quantified on a phosphorimager (ImageQuant and Phosphorimager, Molecular Dynamics Inc., CA). The relative expression of each mRNA to that of β-actin was used to calculate least square means.

Statistical Analysis

Data from dot blot analyses were analysed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analytical System (SAS, 1988). The statistical model had day and reproductive status as the main effects with residuals as the error term. Values in the text are least square means (LSM) ± standard error of the mean (SEM). Orthogonal contrasts were used to determine differences in mRNA expression among days of the estrous cycle, and stages of pregnancy.

Results

Expression of TIMP-1 mRNA

Bovine luteal tissue showed a major hybridization signal (0.9 kb) with ovine TIMP-1 cDNA probe, and a weaker signal at 3.0 kb (Fig. 5-1). Dot blot
Figure 5-1. Northern blot analysis of luteal TIMP-1 mRNA. The same blot was probed for β-actin that served as a control for the loading and the integrity of the RNA. The major transcript appeared at 0.9 kb and a minor transcript at 3.0 kb.
analysis showed that expression of TIMP-1 mRNA varied across the estrous cycle (Fig 5-2). Orthogonal contrasts indicated that TIMP-1 mRNA levels were lower on days 2-3 compared to the other days examined (days 2-3 vs days 16-17 and 20, $P < 0.05$) (Fig. 5-3).

Figure 5-2. Dot blot analysis of TIMP-1 mRNA. Ten µg total RNA isolated from luteal tissue during the estrous cycle (days 2-3, 16-17, and 20) and pregnancy (days 17, 90-120, 170-180, and > 215) was loaded per sample. RNA blots were hybridized with $^{32}$P-labelled TIMP-1 cDNA at 42 C.
There was no difference ($P < 0.34$) in TIMP-1 mRNA expression between days 16-17 and day 20 of the estrous cycle. Expression of TIMP-1 mRNA varied with stage of pregnancy, reaching its highest level during late pregnancy when compared with the other days of pregnancy examined ($P < 0.007$) (Fig. 5-3). Expression of TIMP-1 mRNA was not different between days 16-17 of the estrous cycle and day 17 of pregnancy.

Figure 5-3. TIMP-1 mRNA expression relative to β-actin is presented as LSMean ± SEM ($n = 2-5$) and differences determined by orthogonal contrasts. Days 2-3 vs days 16-17, 20 cycle ($P < 0.05$). Days 17, 90-120, 170-180 pregnant vs days > 215 pregnant ($P < 0.001$). Days 17, 90-120 vs days 170-180, > 215 pregnant ($P < 0.03$).
Expression of TIMP-2 mRNA

Northern blot analysis revealed two transcripts (1 kb and 3.5 kb) of TIMP-2 mRNA following hybridization with an ovine TIMP-2 cDNA probe (Fig. 5-4).

Figure 5-4. Northern blot analysis of luteal TIMP-2 mRNA. The same blot was probed for β-actin to act as a control for the loading and integrity of RNA. The major transcript appeared at 1.0 kb and a minor transcript at 3.5 kb. The 1.0 kb-transcript was predominant.
Results of dot blot analysis (Fig. 5-5) showed a strong effect of day on expression of TIMP-2 mRNA across the estrous cycle ($P < 0.004$) (Fig. 5-6).

Figure 5-5. Dot blot analysis of luteal TIMP-2 mRNA. Ten $\mu$g total RNA isolated from luteal tissue during the estrous cycle (days 2-3, 16-17, and 20) and pregnancy (days 17, 90-120, 170-180, and > 215) was loaded per sample. RNA blots were hybridized with $^{32}$P-labelled TIMP-2 cDNA at 42 C.
Levels of TIMP-2 mRNA were lower on days 2-3 than on the other days examined (days 2-3 vs days 16-17 and 20, \( P < 0.002 \)) (Fig. 5-6).

![TIMP-2 mRNA expression](image)

Figure 5-6. TIMP-2 mRNA expression relative to \( \beta \)-actin is presented as LSMean ± SEM (n = 2-5) and differences determined by orthogonal contrasts. Days 2-3 vs days 16-17, 20 cycle (\( P < 0.002 \)). Day 17 vs days 90-120 (\( P < 0.10 \)). Day 170-180 vs day > 215 (\( P < 0.12 \))
No difference ($P < 0.17$) was observed in expression of TIMP-2 mRNA between day 16-17 and day 20 of the estrous cycle. Although expression of TIMP-2 mRNA did not change overall across pregnancy ($P < 0.20$), orthogonal contrasts indicated that expression tended to be lower during mid-pregnancy when compared with early ($P < 0.10$) and late ($P < 0.12$) pregnancy. The level of expression of TIMP-2 mRNA was similar between days 16-17 of the cycle and day 17 of pregnancy.

**Discussion**

The presence of mRNA for TIMP-1 and TIMP-2 within bovine luteal tissue in the present study confirms earlier observations of synthesis of these proteins by this tissue following incubations with $^3$H-leucine (Ndikum-Moffor et al., 1995). Previous studies have shown that TIMP-1 mRNA is expressed within CL of the cow (Freudenstein et al., 1990; Juengel et al., 1994), ewe (Smith et al., 1994), sow (Tanaka et al., 1992), rat (Mann et al., 1993), and mouse (Nomura et al., 1989).

In the present study, a 0.9 kb-TIMP-1 mRNA transcript was predominant, and a minor transcript was observed at 3.0 kb. The 0.9 kb-transcript is the only size reported in the studies mentioned above. However, M. F. Smith (personal communication) has also observed two TIMP-1 mRNA transcripts in the bovine CL. There was a significant effect of day on the expression of TIMP-1 and TIMP-2 mRNA during the estrous cycle, with levels higher during late than early
luteal phase. Expression of TIMP-1 mRNA was similar between the first and second trimesters, and increased dramatically during the third trimester of pregnancy. These results agree with previous observations of greater synthesis of TIMP-1 protein during the late luteal phase of the estrous cycle, and during late pregnancy (Ndikum-Moffor et al., 1995), indicating that synthesis of TIMP occurs because of the presence of specific TIMP mRNA. However, these results differ from those of Freudenstein et al. (1990) who observed highest expression of TIMP-1 mRNA on days 1-4 and 11-17, low levels on days 5-10 and 18-20 of the bovine cycle, and no expression after day 60 of gestation. In the ovine CL, levels of TIMP-1 mRNA did not vary across the estrous cycle (Smith et al., 1994). We are unable to offer a reason for the major discrepancies between the high levels of expression of TIMP-1 mRNA early in the estrous cycle, no expression after day 60 of pregnancy reported by Freudenstein et al. (1990) in contrast to the lowest expression of TIMP-1 mRNA early in the estrous cycle, and high expression late in pregnancy that was observed in this study.

Northern blot analysis revealed two TIMP-2 mRNA species (approximately 1.0 kb and 3.5 kb) within bovine luteal tissue, similar to that reported for the ewe (Smith et al., 1995), rat (Santoro et al., 1994), and human (Stetler-Stevenson et al., 1990). However, unlike in human, the 1.0 kb-transcript was predominant. Expression of TIMP-2 mRNA varied across the cycle, and was highest on day 16-17. Contrary to these results, Smith et al. (1995) reported in sheep a greater concentration of TIMP-2 mRNA during the early
(days 3 and 7) than the late luteal phase (day 16), and Waterhouse et al. (1993) observed an increase in TIMP-2 mRNA in the rat ovary as pregnancy progressed. These differences may reflect species specificity in the synthesis of TIMP, and may indicate differential roles. The role of TIMP in the CL has not been clearly defined. However, tissue inhibitors of metalloproteinases are specific inhibitors of enzymes that degrade protein components of the extracellular matrix. Additionally, TIMP-1 promotes growth of a wide range of bovine and human cells (Hayakawa et al., 1992), and both TIMP-1 and TIMP-2 have been shown to possess erythroid-potentiating activity (Stetler-Stevenson et al., 1992). Thus TIMP-1 and TIMP-2 may be involved in remodeling of luteal tissue during luteal development as well as at the time of its regression.

Factors regulating expression of mRNA of TIMP-1 and TIMP-2 have not been fully identified. However, evidence suggests that TIMP-1 and TIMP-2 are regulated through different mechanisms (Waterhouse et al., 1993). Expression of TIMP-1 has been shown to increase following the gonadotropin surge in the ewe (Smith et al., 1993; 1994), cow (Freudenstein et al., 1990) and rat (Reich et al., 1991). Mann et al. (1991) also reported that expression of TIMP-1 mRNA in rat ovarian granulosa cells is stimulated by luteinizing hormone, phorbol ester (TPA), and cAMP. The effects of TPA and LH were additive suggesting that each acts through separate intracellular second messenger systems (Mann et al., 1991). Follicle stimulating hormone (FSH) also increases the activity of TIMP-1 and TIMP-2, and expression of their respective mRNA in prepubertal rat
Sertoli cells (Ulisse et al., 1994). Progesterone has also been shown to increase the production of TIMP-1 and TIMP-2 by rabbit uterine cervical fibroblasts in culture (Imada et al., 1994).

The cell types responsible for the synthesis of TIMP-1 and TIMP-2 in the bovine CL have not been determined. In sheep, TIMP-1 mRNA is present in isolated small and large steroidogenic luteal cells and in cells within the luteal connective tissue, with greater expression observed in the large cells (Smith et al., 1994). Similarly, TIMP-2 mRNA is expressed by isolated small and large ovine luteal cells, and other cells within luteal tissue (Smith et al., 1995). TIMP-1 and TIMP-2 mRNA are expressed by many cell types including fibroblasts, monocytes, and macrophages (Stetler-Stevenson et al., 1990). Apart from the steroidogenic large and small luteal cells, the CL also contains monocytes, macrophages, endothelial cells and fibroblasts. Thus the non-steroidogenic luteal cells may also contribute to luteal synthesis of TIMP-1 and TIMP-2.

The role of TIMP in luteal function is not yet understood but has been suggested to be involved in tissue remodelling during formation of the CL following ovulation. However, the role of TIMP-1 and TIMP-2 in the CL during pregnancy has not been discussed. In the present study, expression of TIMP-1 mRNA and TIMP-2 mRNA was highest during the late luteal phase of the estrous cycle and late pregnancy. TIMP-1 may act in an autocrine and/or paracrine fashion to maintain steroidogenesis and CL function during the estrous cycle and pregnancy, and control tissue remodelling during involution and regression.
CHAPTER 6
CHANGES IN THE EXPRESSION OF MESSENGER RIBONUCLEIC ACID FOR
MANGANESE SUPEROXIDE DISMUTASE IN THE BOVINE CORPUS LUTEUM
DURING THE ESTROUS CYCLE AND PREGNANCY

Introduction

Results from a previous experiment (chapter 3) showed that the bovine
corpus luteum synthesizes and releases manganese superoxide dismutase (Mn
SOD) in culture. Manganese SOD is one of three superoxide dismutases,
including copper SOD and zinc SOD. Manganese SOD is encoded by a nuclear
gene and is found predominantly in the mitochondria (Church, 1990).
Superoxide dismutases are believed to protect cells from reactive oxygen
species (such as superoxide anions) which may damage the cells. Superoxide
dismutase catalyses the conversion of superoxide anions to hydrogen peroxide
and oxygen; \(2\text{O}_2^- + 2\text{H}^+ = \text{H}_2\text{O}_2 + \text{O}_2\), and has been referred to as a scavenger
of superoxide radicals. Damage to luteal cells by reactive oxygen species has
been proposed as one of the mechanisms of luteal regression (Riley and
Behrman, 1991). Production of Mn SOD can be stimulated by oxidants,
cytokines, and lipopolysaccharides (Melendez and Baglioni, 1993). Laloraya et
al. (1988) observed that LH induces production of superoxide dismutase,
suggesting a role of SOD in luteal function. It was also observed that levels of
ovarian superoxide dismutase and superoxide radicals change inversely during the rat estrous cycle. Prostaglandin F\textsubscript{2\alpha} is the natural luteolysin in many domestic species. Treatment of rats with prostaglandin F\textsubscript{2\alpha} \textit{in vivo} has been shown to cause a rapid transient increase in superoxide radical production and a decrease in fluidity of plasma membranes from luteinized rat ovaries (Sawada and Carlson, 1991). In a later study Sawada and Carlson (1994) suggested that superoxide radicals may disrupt LH-stimulated progesterone secretion by rat CL. Sato et al. (1992) showed that treatment of rats with hCG causes a decrease in ovarian superoxide dismutase activity, especially that of Mn SOD, and an increase in expression of Mn SOD mRNA. However, Laloraya et al. (1988) observed a transient increase (declined by 60 min post-PGF injection) in ovarian total SOD activity, but the changes specific to Mn SOD were not discussed. Sato et al. (1992) also observed that long-acting SOD blocks hCG-induced ovulation while H\textsubscript{2}O\textsubscript{2}-inactivated SOD has no effect, and suggested the inhibition could be due to dismutation of superoxide radicals by SOD. Rueda et al. (1995) proposed that the increased oxidative stress during luteolysis was due to reduced activity of enzymes that metabolize superoxide radicals. This hypothesis was supported by observations that functional bovine CL (day 20 of pregnancy) expresses significantly higher levels of mRNA encoding Mn SOD than regressed CL (day 20 of the estrous cycle) (Rueda et al., 1995).

Little is known about the pattern of expression of Mn SOD during the bovine estrous cycle and pregnancy. The objective of the present study was to
examine the changes in expression of mRNA encoding Mn SOD across day of
the cycle and during pregnancy.

Materials and Methods

Tissue Collection

Angus and Hereford crossbred cows were used for the study. Estrus (day 0) was determined by observing cows twice daily for estrous behavior with
assistance of a bull surgically modified to be incapable of mating. Cows in the
pregnant group were bred by artificial insemination at observed estrus. Day 17
pregnancy was confirmed by the presence of an embryo in flushings from the
uterus. Later stages of pregnancy were estimated by measurement of crown-
rump length of the calves (Winters et al., 1942). Reproductive tracts were
collected within 5 min after exsanguination from cows on days 2-3 (n = 3), 7 (n =
1), 16-17 (n = 4) and 20 (n = 2) of the estrous cycle, and on days 17 (n = 5), 90-
120 (n = 2), 170-180 (n = 3) and > 215 (n = 5) of pregnancy. The CL was
dissected from surrounding ovarian stroma, snap-frozen in liquid nitrogen, and
then stored at -80 C until further analysis.

Isolation of RNA

Total cellular RNA was isolated from luteal tissue by the acid guanidinium
thiocyanate-phenol-chloroform extraction method (Puissant and Houdebine,
1990). Fresh or frozen tissue (1 g) was homogenized on ice in a solution containing 10 ml 4 M cold guanidinium thiocyanate, 1 ml 2 M sodium acetate (pH 5.0) and 78 µl 2-mercaptoethanol, using a polytron tissue homogenizer (Tissumizer®, Tekmar Company, Cincinnati, OH). Total RNA was extracted from homogenate with a phenol-chloroform mixture (10 ml:2 ml) and centrifuged at 4000 x g for 15 min. The upper phase was treated with isopropanol and centrifuged to precipitate the RNA. The pellet was resuspended in 4 M lithium chloride, repelleted by centrifugation, and dissolved in RNA buffer (10 mM Tris, 1 mM EDTA, 0.5% (w/v) SDS, pH 7.5). The RNA in solution was treated with chloroform and the upper phase collected, treated with 2 M sodium acetate and isopropanol, and stored at -80 C to reprecipitate the RNA. Pure RNA pellet was retrieved after centrifugation at 4000 x g for 10 min and dissolved in sterile water. The concentration and purity (260 : 280 nm ratio) of the RNA were determined. Integrity of the RNA preparations was assessed by electrophoresis on a 1.2% (w/v in 1X TAE buffer) agarose gel. Total RNA samples were stored at -80 C until further analysis.

**Restriction Analysis and Isolation of Plasmid DNA Insert**

Plasmid DNA containing human Mn SOD cDNA was a gift from Dr. Harry Nick of the Department of Biochemistry and Molecular Biology, University of Florida. The plasmid DNA (pUc 19) was digested with EcoR1 at 37 C for 16 h. Plasmid digests were separated by electrophoresis on a 1.2% agarose gel
stained with ethidium bromide. The fragments were transferred by electrophoretic blotting to DEAE cellulose membrane (Schleicher and Schuell, Keene, NH), and then extracted from membrane with a high salt buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8). The extracted insert was reprecipitated with 3 M sodium acetate and 100% ethanol, then centrifuged at 12,000 x g to repellet. The DNA pellet was freeze dried and dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The size of the purified insert was checked on a 1.2% agarose gel by comparing with a series of RNA markers. Digestion of plasmid DNA containing Mn SOD cDNA yielded one fragment about 1.5 kb in size.

**Northern Hybridization**

Size of mRNA for Mn SOD in luteal tissue was determined by northern blotting (Sambrook et al., 1989). Total RNA (30 μg) was denatured in denaturing buffer (24 mM HEPES, 6 mM sodium acetate, 1.2 mM EDTA, 50% (v/v) deionized formamide, 2.2 M formaldehyde) for 1 h on ice, followed by incubation at 65 C for 15 min. Denatured samples were separated by electrophoresis at 20 volts for 16 h on a 1.5% agarose-formaldehyde gel in 1X running buffer (0.5 M NaH₂PO₄, 0.5 M Na₂HPO₄, pH 7). The gels were stained with ethidium bromide, and the presence of distinct bands of the 28 s and 18 s ribosomal RNA was used to assess the integrity of the RNA.

Fractionated RNA was transferred by capillary blotting to nylon membrane (Biotrans, ICN, Irvine, CA) using the TurboBlotter™ (Schleicher and Schuell) in
20X SSC (single-strength SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Filters were exposed to ultraviolet light for 90 sec to immobilize the RNA, then prehybridized for 2 h at 42 C in prehybridization buffer (5X Denhardt, 4X SSC, 0.5 M sodium phosphate pH 6.5, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) followed by hybridization at 42 C for 16 h in buffer (1X Denhardt, 4X SSC, 0.5 M sodium phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) containing $^{32}$P- Mn SOD cDNA (ICN Biomedicals) (10$^6$ cpm /ml of hybridization buffer) labeled by nick translation (Amersham International plc). Blots were washed twice in double-strength SSC-0.1% SDS at 42 C for 15 min each, with shaking, followed by one wash in 0.1-strength SSC-0.1% SDS at 42 C for 10 min.

**Autoradiography**

Blots were exposed to x-ray film (Kodak XAR, Eastman Kodak) and an intensifying screen at -80 C to detect hybridization signals. For hybridization with a different probe, blots were washed with hot 1% SDS for 1 h. In this study, blots were rehybridized with a β-actin cDNA probe at 42 C to assess the amount and integrity of total RNA loaded in each lane.
**Dot Blot Hybridization**

Total RNA (10 μg) was denatured in denaturation buffer (20 mM Tris, pH 7.0, 50% formamide, 6% formaldehyde), followed by incubation at 65°C for 5 min. The denatured samples were immobilized on membrane (Biotrans, ICN) using a microsample filtration unit (Schleicher and Schuell). The filters were exposed to ultraviolet light for 90 sec to crosslink the RNA to the membrane, then prehybridized in buffer (5X Denhardt, 4X SSC, 0.5 M sodium phosphate pH 6.5, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) at 42°C for 2 h, followed by hybridization in buffer (1X Denhardt, 4X SSC, 0.5 M sodium phosphate, 0.1% SDS, yeast RNA (0.25 mg/ml), 50% (v/v) formamide) containing the \(^{32}\)P-Mn SOD cDNA (106 cpm/ml of hybridization buffer) labeled by nick translation (Amersham International plc) at 42°C for 16 h. The blots were washed twice with 2X SSC, 0.1% SDS at 42°C for 15 min each on a shaker, followed by two washes with 0.1X SSC, 0.1% SDS at 42°C for 15 min each. The blots were exposed to x-ray film (Eastman Kodak) and/or phosphorimaging cassettes, and hybridization signals were detected and quantified on a phosphorimager (ImageQuant and Phosphorimager, Molecular Dynamics Inc.). The relative expression of each mRNA to that of ß-actin was used to calculate least square means.
**Statistical Analysis**

Data from dot blot analyses were analysed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analytical System (SAS, 1988). The statistical model had day and reproductive status as the main effects with residuals as the error term. Values in the text are least square means (LSM) ± standard error of the mean (SEM). Orthogonal contrasts were used to determine differences among days of the cycle, and stages of pregnancy.

**Results**

**Northern Blot Analysis**

Northern blot analysis revealed the presence of three mRNA transcripts of approximately 1.5, 1.9, and 3.7 kb in size in bovine luteal tissue. The 1.5 kb-transcript was the most predominant (Fig. 6-1).

**Dot Blot Analysis**

Dot blot analysis showed that levels expression of Mn SOD mRNA varied across the estrous cycle \((P < 0.06)\), and orthogonal contrasts indicated that levels were higher during the late compared to the early luteal phase of the estrous cycle \((P < 0.01)\) (Fig. 6-2).
Figure 6-1. Northern blot analysis of luteal Mn SOD mRNA. The same blot was probed for β-actin that served as a control for the loading and the integrity of the RNA. The major transcript appeared at 1.5 kb and two minor transcripts at 1.9 kb and 3.7 kb.
Figure 6-2. Dot blot analysis of Mn SOD mRNA. Total RNA (10 μg) isolated from luteal tissue during the estrous cycle (days 2-3, 16-17, and 20) and pregnancy (days 17, 90-120, 170-180, and > 215) was loaded per sample. RNA blots were hybridized with 32P-labelled Mn SOD cDNA at 42 C. Expression on days 2-3 < days 16-17 and 20 (P < 0.01).
Although expression of Mn SOD mRNA did not vary overall across pregnancy ($P < 0.27$), orthogonal contrasts showed that levels were lower during early pregnancy compared to the other days of pregnancy examined ($P < 0.07$).

**Discussion**

Results of this study confirmed synthesis of manganese superoxide dismutase by bovine CL as indicated in an earlier study (Ndikum-Moffor et al., 1995). Bovine luteal tissue expressed three mRNA transcripts of approximately 1.5, 1.9, and 3.7 kb following hybridization with human Mn SOD cDNA. The 1.5 kb-transcript was the most predominant, followed by the 3.7 kb-transcript. Melendez and Baglioni (1993) observed two Mn SOD mRNA transcripts (1.0 kb and 4.0 kb) in human cell lines following TNF-alpha-induced expression; the 4-kb mRNA is less stable and has a shorter half-life than the 1-kb mRNA. Three Mn SOD mRNA transcripts of similar sizes as observed in the present study have been reported in luteal tissue of the cow (Rueda et al., 1995) and rat (Sato et al., 1992).

Manganese SOD is believed to play a role in ovulation (Laloraya et al., 1988; Sato et al., 1992) and luteal regression (Riley and Behrman, 1991; Sawada and Carlson, 1994; Rueda et al., 1995). Superoxide dismutase has been shown to inhibit vascular permeability of injured tissues (Ando et al., 1990), while hCG has been observed to increase vascular permeability of the ovary. Thus SOD is thought to inhibit hCG-induced ovulation in the rat by dismutation.
of superoxide radicals, which may be responsible for the increase in vascular permeability, promoting disorganization of cells of follicular walls and ovulation (Sato et al., 1992).

In the present study changes in the expression of Mn SOD mRNA in the corpus luteum (post-ovulation) revealed higher levels of expression during the late than early luteal phase of the estrous cycle. Manganese SOD is produced by the mitochondria; thus changes in expression of Mn SOD mRNA may reflect changes in the mitochondrial status of luteal cells. The large luteal cell of the cow contains an abundance of mitochondria which swell and take on dense inclusions as early as day 14 of the estrous cycle (Fields et al., 1992). In this study, lower expression of Mn SOD mRNA was detected on day 2-3 (when no swelling of mitochondria is present), and greatest expression during the late luteal phase when swelling of mitochondria is at its highest (Fields et al., 1992). An increase in expression of Mn SOD mRNA during the involutive phase of the estrous cycle agrees with increased synthesis and release of Mn SOD protein observed at this time (Ndikum-Moffor et al., 1995). This may serve as a defense mechanism by the CL to counteract the first stages of luteal regression caused by reactive oxygen species. This is plausible since by day 20 of the estrous cycle, the CL is completely regressed and levels of Mn SOD mRNA decline (Rueda et al., 1995). Evidence for the possible role of Mn SOD in preventing luteal regression was provided in a study by Rueda et al. (1995) in which regressed bovine CL (day 20 of the estrous cycle) exhibited lower expression of
Mn SOD mRNA as opposed to functional CL (day 20 of pregnancy).

Little has been reported about changes in expression of Mn SOD mRNA during pregnancy. Results of this study revealed that expression of Mn SOD mRNA did not vary overall across stages of pregnancy, although levels of expression tended to be lower during early pregnancy compared to the other stages examined. Maintenance of the CL at that time is crucial to maintain pregnancy (Niswender and Nett, 1994). Thus manganese SOD mRNA could be one of many genes expressed during pregnancy to counteract a luteal insult of PGF2-alpha that might preempt pregnancy. It is interesting that in the day 14 to day 17 pregnant cow, mitochondria of the large luteal cells do not swell and are devoid of dense inclusions (Fields et al., 1992). Thus changes in Mn SOD gene expression may be indicative of luteal function during the estrous cycle and pregnancy.
CHAPTER 7
GENERAL DISCUSSION AND CONCLUSIONS

Adequate luteal function in cattle is important for control of the estrous cycle, the maintenance of pregnancy (Tanabe, 1966; Chew et al., 1979), and normal parturition and expulsion of fetal membranes (Estergreen et al., 1967; Pimentel et al., 1987). Identification of intraluteal factors that may be involved in regulation of luteal function will be useful in the management of cows with suboptimal luteal function and low fertility rates. Histological studies have shown that the CL has the structural machinery required for synthesis and secretion of proteins (Fields et al., 1985; 1992). Apart from its role in progesterone synthesis, recent studies indicate that the CL synthesizes proteins and peptide factors which may act in an autocrine and/or paracrine manner to regulate luteal and ovarian function (Schams, 1989). Objectives of this dissertation were to investigate de novo protein synthesis by luteal explants in culture, determine the pattern of synthesis and release during the estrous cycle and pregnancy, identify and characterize the newly-synthesized proteins, and determine presence and pattern of expression of their respective mRNA during the estrous cycle and pregnancy.
In experiment 1, the protein synthetic capability of the CL was investigated during the estrous cycle (days 3, 7, 11, 14, 17 and 19) and pregnancy (days 17, 88, 170, and 240 or greater). Luteal explants were incubated with $^3$H-leucine and the level of incorporation of the radiolabel into proteins was measured by estimating percent incorporation and TCA-precipitable radioactivity (chapter 3). Data from this experiment showed that the bovine CL is capable of incorporating amino acids for de novo protein synthesis in vitro during the estrous cycle and pregnancy. In general, the synthetic ability of the CL did not vary with reproductive status as percent incorporation was similar between day 17 of the estrous cycle and day 17 of pregnancy. To determine the pattern of protein synthesis and release, proteins released into luteal-conditioned medium were concentrated by dialysis and lyophilization, and were then separated by IEF followed by 2D-PAGE. Gels were analyzed by fluorography. Analysis of fluorographs showed that the CL synthesized many proteins in culture. For convenience, the major proteins were numbered from 1 to 11. The newly-synthesized proteins had molecular weights ranging from 12 to > 200 kDa. The percent incorporation of radiolabel into protein varied across the estrous cycle and pregnancy. Fewer proteins were synthesized by the young CL of day 3 than by CL of the other days of the cycle examined. As the cycle progressed, the CL seemed to attain its full protein-synthetic ability on day 11 since all the major proteins synthesized were present on day 11. Although the CL on day 3 synthesized fewer proteins, it was unique in its synthetic profile
since the proteins observed on day 3 (proteins 1-4) were not observed on the other days of the cycle and during pregnancy. Similarly, proteins synthesized on the other days of the cycle and pregnancy were not observed on day 3 (chapter 3).

Following identification of newly-synthesized proteins by fluorography, the next phase of experiment 1 was to further characterize the proteins by N-terminal amino acid sequence analysis. Proteins separated by 2D-PAGE were transferred to PVDF membrane and subjected to N-terminal amino acid sequencing. Protein 1 (35 kDa, pl 5.5) was identified as apolipoprotein E (Apo E), protein 8 (30 kDa, pl 8.0) was identified as tissue inhibitor of metalloproteinases-1 (TIMP-1), protein 9 (20 kDa, pl 8.0) as TIMP-2, protein 10 (22 kDa, pl 8.0) as manganese superoxide dismutase (SOD), and protein 11 (27 kDa, pl 6.0) as Apo A-1. Luteal synthesis of TIMPs has been reported in the ewe (Smith et al., 1993; 1994), cow (Freudenstein et al., 1990; Juengel et al., 1994), sow (Tanaka et al., 1992), rat (Mann et al., 1993) and mouse (Nomura et al., 1989). However, this is the first study to evaluate synthesis of TIMP-1 by the bovine CL throughout gestation. To our knowledge, luteal synthesis of Apo A-1 has not been reported in any species and this is the first report of Apo E synthesis by the bovine CL. Studies in the rat showed that Apo E is produced predominantly by theca cells of the ovarian follicle, and synthesis of Apo E was not observed in the corpus luteum (Polacek et al., 1992). However, Nicosia et al. (1992) reported presence of Apo E mRNA in rat CL.
In the present study (experiment 1, chapter 3), synthesis of Apo E was observed only on day 3 of the estrous cycle, was not observed on the other days of the cycle examined and during pregnancy. Similarly, expression of Apo E mRNA was detected within CL only on days 2 and 3 of the estrous cycle (experiment 2, chapter 4). No Apo E mRNA expression was detected on the other days of the cycle examined and during pregnancy. Thus luteal synthesis of Apo E protein during the early luteal phase (day 3) was confirmed by the presence of Apo E mRNA at that period of the estrous cycle, and the absence of Apo E during the other periods of the cycle examined and pregnancy was due to absence of its mRNA at those times. A single mRNA transcript (1.0 kb) in bovine luteal tissue hybridized with the Apo E cDNA probe. The size of the transcript was similar to that expressed in rat ovarian follicles (Polacek et al., 1992).

The significance of synthesis of Apo E only during the early luteal phase of the estrous cycle is not known, but may suggest presence of a regulatory (stimulatory) factor at that time which is absent or inactivated during the remaining part of the cycle and during pregnancy. A good candidate would be the gonadotropins and agents that activate adenylate cyclase and protein kinase A, which have been shown to increase production of Apo E (Driscoll et al., 1985) and Apo E mRNA (Polacek et al., 1992) in rat granulosa cells. Driscoll et al. (1985) showed that FSH stimulates secretion of newly-synthesized Apo E by granulosa cells in culture, in a dose- and time-dependent manner, and the
effects of FSH were mediated through cAMP. However, as the granulosa cells differentiated in culture, they lost responsiveness to FSH and cAMP and Apo E mRNA was not expressed in luteinized granulosa cells (Driscoll et al., 1985).

The preovulatory surge of gonadotropins may stimulate Apo E synthesis observed on days 2 and 3 of the estrous cycle, and ceases during diestrus and pregnancy when plasma levels of gonadotropins are low or negligible. The role of Apo E in the CL is not known. However, Apo E is a constituent of VLDL, LDL, and HDL, and has been shown to mediate binding of lipoproteins to the LDL receptor (Mahley, 1988). Thus luteal Apo E may have an autocrine and/or paracrine role to provide cholesterol for membrane biosynthesis and cell proliferation at a time when the CL is undergoing rapid reorganization and growth following the trauma of ovulation. A similar role of Apo E in nerve regeneration following injury has been suggested (Mahley, 1988).

Apo A-1 was not observed on days 3 and 7, but was observed on the other days of the estrous cycle examined and throughout pregnancy (experiment 1). This is a novel finding because to our knowledge, there has been no report on luteal synthesis of Apo A-1 in any species. Apo A-1 synthesized and released by luteal explants in this study shared 100% amino acid sequence identity (31 residues) with bovine Apo A-1 (O'hUigin et al., 1990). Synthesis of Apo A-1 by bovine luteal tissue was confirmed by results from experiment 2 (chapter 4) which showed that bovine luteal tissue expressed Apo A-1 mRNA. Bovine luteal tissue exhibited a single hybridization signal (1.0 kb) with a human
Apo A-1 cDNA probe. In agreement with the protein data in experiment 1, northern blot analysis showed negligible expression of Apo A-1 mRNA during the early luteal phase (days 2 and 3) of the estrous cycle (experiment 2). However, dot blot analysis indicated expression of Apo A-1 mRNA on days 2 and 3 and levels did not differ when compared with the other days of the estrous cycle examined (experiment 2). Newly-synthesized Apo A-1 released in culture was similar among days 11, 14 and 17 of the estrous cycle, but increased on day 19. Thus there appears to be translational regulation of the Apo A-1 mRNA in favor of greater protein synthesis during the late luteal phase of the estrous cycle as observed in experiment 1. Although there was no difference overall in the synthesis and release in culture of Apo A-1 by CL across pregnancy (experiment 1), levels tended to be greater on day 170 when compared with the other days of pregnancy examined. Expression of Apo A-1 mRNA varied with stage of pregnancy and was higher during the second than the first half of gestation (chapter 4).

Apo A-1 is produced mainly by the liver and small intestine, but is also synthesized by many tissues in humans and several animal species (O'hUigin et al., 1990; Sparrow et al., 1992). However, there have been no reports on luteal synthesis. The present study has shown for the first time that a CL synthesizes Apo A-1. The role of Apo A-1 in the CL is not known at this point. Apo A-1 is the major protein component of HDL. High density lipoprotein is more important in vivo in the cow than LDL because of its higher concentrations in blood. In
addition, it has been shown that HDL is required to maintain bovine luteal cells in culture, and HDL is utilized by luteal cells as a source of cholesterol for steroidogenesis (Pate and Condon, 1989). Thus luteal Apo A-1 may play an autocrine and/or paracrine role in the CL and ovary to provide cholesterol for steroidogenesis, since HDL is known to enhance synthesis of progesterone by bovine luteal cells in culture (Pate and Condon, 1989). There is evidence that a female with a functional CL has higher levels of serum Apo A-1 than an anestrous female (Oikawa and Katoh, 1995). However, the contribution of luteal Apo A-1 to circulating levels is unknown and is under investigation. Sorci-Thomas et al. (1988) suggested that Apo A-1 produced by peripheral tissues may not contribute significantly to the plasma Apo A-1 pool but may play a role (autocrine and/or paracrine) in lipid metabolism within the tissue of secretion.

Kushwaha et al. (1990) showed that Apo A-1 content of HDL is greater in baboons treated with a combination of estradiol and progesterone, compared to those treated with estradiol alone. Treatment with progesterone alone had no effect on Apo A-1 synthesis (Kushwaha et al., 1990). Although the effects of progesterone on luteal synthesis of Apo A-1 was not specifically investigated in this study, changes were observed in plasma progesterone across days of the estrous cycle in this study (experiment 1), with no synthesis of Apo A-1 on days 3 and 7, elevated synthesis on day 19 (experiment 1), and similar expression of Apo A-1 mRNA (experiment 2) across days of the estrous cycle examined. Thus luteal synthesis of Apo A-1 does not seem to depend on circulating levels of
progesterone. It would be useful to identify the cell types responsible for the synthesis of Apo A-1, as this may give an indication of its role in the corpus luteum.

Results from experiment 1 also showed that the bovine CL synthesized and released tissue inhibitor of metalloproteinases-1 and -2 in culture during the estrous cycle and pregnancy. Synthesis and release in culture of TIMP-1 was absent on day 3, and increased on day 19 of the estrous cycle. Luteal synthesis of TIMP-1 and TIMP-2 was confirmed in experiment 3 (chapter 5) by the presence of mRNA for TIMP-1 and TIMP-2 within bovine luteal tissue. Bovine luteal tissue showed a major hybridization signal at 0.9 kb and a weaker signal at 3.0 kb (experiment 3). Expression of TIMP-1 mRNA was highest during the late luteal phase of the estrous cycle and increased with advancing pregnancy (experiment 3). Synthesis of TIMP-1 followed a similar pattern with expression of its mRNA during the estrous cycle. Although no significant differences in synthesis of TIMP-1 were observed during pregnancy, levels tended to be greater during late pregnancy (day 240 or greater), and similarly, expression of TIMP-1 mRNA was significantly greater during late pregnancy (day 215 or greater) when compared with the other days of pregnancy examined.

Northern blot analysis also revealed two TIMP-2 mRNA transcripts (1.0 kb and 3.5 kb) following hybridization to ovine TIMP-2 cDNA probe similar to that reported for the ewe (Smith et al., 1995), rat (Santoro et al., 1994), and human (Stetler-Stevenson et al., 1990). Similar to the ewe (Smith et al., 1995) but
unlike in the human, the 1.0 kb-TIMP-2 transcript was predominant in this study. In contrast to the changes observed in this study Smith et al. (1994) observed no differences in expression of TIMP-1 mRNA during the ovine estrous cycle. Another contradiction is Freudenstein et al. (1990) observed high TIMP-1 mRNA expression during the early luteal phase of the bovine estrous cycle, and no expression after day 60 of gestation. We are unable to offer a reason for the discrepancies between this study and that of Freudenstein et al. (1990) conducted with bovine tissue. However, our observations of elevated expression of TIMP-1 mRNA during regression agrees with those reported by Juengel et al. (1994). This is the first study to describe the changes in expression of TIMP-1 and TIMP-2 mRNA during pregnancy in the cow.

The function(s) of TIMP-1 and TIMP-2 in the CL has not been defined, but it has been suggested that TIMPs may be involved in tissue remodelling during formation of the CL following ovulation (Smith et al., 1994). However, in the present study, TIMP-1 was synthesized by bovine luteal tissue and expression of its mRNA increased as the CL matured and regressed. TIMP-1 may serve in an autocrine and/or paracrine manner to maintain steroidogenesis (Boujrad et al., 1995) and CL lifespan by preventing proteolytic digestion of the extracellular matrix of the CL. The large increase during luteal regression is consistent with an orderly demise of the CL (Juengel et al., 1994).

In experiment 1 (chapter 3) and experiment 4 (chapter 6) data was presented to show that the bovine CL synthesized manganese SOD during the
estrous cycle and pregnancy. During the estrous cycle, expression of manganese SOD mRNA was lower during the early luteal phase than at the time of luteal involution (day 16-17) and regression (day 20). During pregnancy, levels of manganese SOD mRNA were lower during early pregnancy compared to the other days of pregnancy examined. There is a paucity of information on the temporal changes in gene expression and activity of manganese SOD in the CL during the reproductive cycles. Superoxide dismutase isoenzymes are scavengers of reactive superoxide anions and thus may protect cells from oxidative damage. Manganese SOD has been implicated as involved in ovulation and luteal regression. Laloraya et al. (1988) showed that activity of total superoxide dismutase varies inversely with levels of superoxide anion in the rat ovary during the estrous cycle. The SOD activity contributed by manganese SOD was not discussed in that study. Sato et al. (1992) showed that ovarian levels of manganese SOD mRNA increase after treatment of rats with hCG, and demonstrated that manganese SOD inhibited hCG-induced ovulation. Results from experiment 1 and 4 of this dissertation showed an increase in manganese SOD mRNA at a time of the estrous cycle (day 16) when the CL is entering a regressive phase, and a decline at time of complete regression (day 20). An increase manganese SOD during the involutive phase of the estrous cycle preceeding regression may serve as a mechanism by the CL to counteract the first stages of luteal regression caused by reactive oxygen species. A fully regressed CL (day 20 of the bovine estrous cycle) has been shown to express
significantly lower levels of manganese SOD mRNA when compared to a functional CL (day 20 of pregnancy) (Rueda et al., 1995). Luteal expression of manganese SOD mRNA during the estrous cycle may indicate a CL undergoing an insult. Expression during pregnancy may reflect a rescue effect.

It was observed in this study that synthesis and release in culture of all the proteins discussed, except the TIMP-2/SOD complex, did not differ quantitatively between day 17 of the estrous cycle and day 17 of pregnancy.

In order to confirm day of the estrous cycle (observed estrus was day 0) total CL weight and plasma progesterone were monitored (chapter 3). As expected, CL weight varied across days of the estrous cycle being lowest on day 3, increased to day 14, and declined thereafter. Plasma progesterone followed a similar pattern and levels declined after day 14 to low levels on day 19, indicative of luteal regression. CL weight and plasma progesterone did not vary across pregnancy starting on day 17. The protein synthetic ability of the CL during luteal regression observed on days 17 and 19 was unexpected and suggests that the CL maintained its protein synthetic ability independently of the decline in steroidogenesis.

Conclusions. In summary, results of the four experiments show that the bovine CL synthesizes many proteins. Eleven proteins were characterized as synthesized and released by the CL across the estrous cycle and pregnancy. Five of these proteins were identified by N-terminal amino acid sequence analysis as Apo E, Apo A-1, TIMP-1, TIMP-2 and Mn SOD. The gene
expression and temporal changes of their respective mRNA across the estrous cycle and pregnancy were also investigated. Two of these proteins, Apo E and Apo A-1 are novel as there have been no reports of their synthesis by the CL. Apolipoprotein E is associated with VLDL and LDL and apolipoprotein A-1 is associated with HDL. HDL and LDL are sources of cholesterol for the cow (Pate and Condon, 1989). Apolipoproteins mediate uptake and binding of lipoproteins to the lipoprotein receptor. Thus they may be involved in regulating cholesterol availability for membrane and progesterone biosynthesis.

Two other proteins identified, (TIMP-1 and TIMP-2) have been reported in luteal tissue of several species including the cow during the estrous cycle. However, this is the first report of these proteins being synthesized and secreted by the CL of the pregnant cow. TIMP are produced by many cell types in several tissues where they regulate degradation of the extracellular matrix (Werb, 1989). Although their role in the CL has not been defined, they are believed involved in tissue remodelling during rapid development of the CL post-ovulation, the demise and reorganization of the CL that would allow for ovulation. We also believe that they play a role in maintaining luteal function during pregnancy as TIMP-1 has been shown to stimulate steroid hormone synthesis in the testis (Boujrad et al., 1995).

Suggested roles of these proteins in luteal function are speculative at this point. Determination of factors that regulate luteal production of these proteins and determination of their functions will be critical to better reproductive
management of the cow and related species. For example, the significance of synthesis of Apo E only during early luteal development (chapter 3 and 4) needs to be investigated; factors regulating Apo E gene expression that are present only during the small physiological window need to be identified. Due to the heterogenous nature of the cellular components of the CL, identification of the cell types responsible for production of these proteins would provide more insight into what their functions might be. Finally, a better understanding of the regulatory role of these proteins on luteal development and function may serve as a model to study factors that regulate tissue growth under normal and pathological conditions in humans.

**ROLE OF LUTEAL PROTEINS**

- Follicle → Ovulation → Young CL → Apo E → Apo A-1
- Young CL: LH, FSH, E2
- Mature CL: Apo E, HDL, Cholesterol
- Regressing CL: TIMP, SOD, Progesterone
- TIMP: Steroidogenesis, Tissue Remodelling
- SOD: Cholesterol

Diagram shows the roles of various proteins and hormones in luteal development and function.
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APPENDIX 1
ANIMAL CARE AND TISSUE COLLECTION

Experimental Protocol

Animals

Forty-eight cross-bred beef cows were used in the study. Estrus (day 0) was determined by monitoring cows twice daily for standing to be mounted by a vasectomized bull. Some cows were bred by artificial insemination at observed estrus. Early pregnancy was confirmed by the presence of an embryo in flushings of the uterus. Later stages of pregnancy were determined by measurement of crown-rump length of the calves (Winters et al., 1942).

Preparation for Tissue Collection

Autoclave all materials to be used. These include:

- Towels
- Forceps
- Scissors
- Stadie Rigg's tissue slicer (Thomas Scientific, Swedesboro, NJ)
- Tissue slicer handle
- Autopipet tips.
- 15 x 100 mm Petri dishes (Fisher Scientific Co., Springfield, NJ)
Individually wrapped sterile disposable serological pipets.

**Preparation of Culture Medium**

The medium was prepared as described by Basha et al. (1980) from Eagle’s Minimum Essential Medium (MEM, Sigma Chemical Co., St. Louis, MO), that was deficient in leucine, methionine and sodium bicarbonate. One liter of stock incomplete MEM was prepared as follows:

1. Empty the bottle containing lyophilized Eagle’s MEM (9.4 g/l) into a 1 l beaker.
2. Then add:
   - Glucose (3 g)
   - Methionine (1.5 mg)
   - Leucine (5.2 mg)
   - Lysine-HCl (72.5 mg) to achieve 4.0, 0.1, 0.1, and 1.0 times, respectively, their usual concentrations in MEM.
   - Sodium bicarbonate (2.2 g)
   - 10 ml non-essential amino acids solution
   - 10 ml vitamins mixture
   - Insulin (200 IU)
3. Adjust pH to 7.1-7.3 with 1 M HCl. Make up to 1 l with deionized water.
4. Filter-sterilize the medium (0.22 μm) into sterile bottles in a laminar flow hood.
5. Store medium at 4 C.
For labeling with $^3$H-leucine, methionine (1.5 mg/100 ml) and antibiotic-antimycotic mixture (ABAM) (1%, v/v) were added to the stock (modified) incomplete MEM to obtain leucine-deficient incomplete MEM. Similarly, for the $^{35}$S-methionine labeling incubations, methionine-deficient medium was prepared by adding leucine (5.2 mg/100 ml) to the stock modified incomplete MEM. For $^3$H-glucosamine culture, incomplete modified MEM was used.

**Blood Collection**

Trunk blood was collected at slaughter into heparinized tubes. Blood samples were centrifuged at 3000 rpm for 15 min at 4 °C. Plasma was collected and stored at -20 °C until analysed for progesterone by radioimmunoassay (see appendix 2).

**Tissue Collection**

Reproductive tracts were obtained from cows within 5 min after exsanguination. The ovary containing the corpus luteum (CL) was collected aseptically from cows on days 3 (n = 4), 7 (n = 3), 11 (n = 4), 14 (n = 5), 17 (n = 3) and 19 (n = 3) of the estrous cycle and from cows of early (day 17, n = 5), first (day 88, n = 5), second (day 170, n = 7), and third (> day 240, n = 9) trimester of pregnancy. The ovaries were harvested and transferred immediately to a sterile Petri dish containing Eagle's incomplete Minimum Essential Medium (modified MEM) pre-warmed at 37 °C. Subsequent processing of tissue was done in a laminar flow hood. The CL was dissected out of the ovaries and trimmed of any excess fat and surrounding ovarian stroma. Each CL was then weighed and
transferred to another Petri dish containing fresh pre-warmed incomplete MEM.

Luteal tissue for RNA isolation were snap-frozen in liquid nitrogen after collection, and then stored at -80°C until further analysis.

**Preparation of Luteal Tissue**

1. Prepare 0.5 mm slices of luteal tissue with the use of sterile forceps and scissors, using the Stadie Rigg's tissue slicer.

2. Wash the slices 3 times with incomplete MEM to reduce serum proteins in the medium during incubation.

3. For culture in each 15 x 100 mm Petri dish, weigh 500 mg of luteal slices directly in the Petri dish containing 15 ml of culture medium (see appendix 3).
APPENDIX 2
PROGESTERONE RADIOIMMUNOASSAY

Introduction

The DPC Coat-A-Count (Diagnostic Products Corporation, Los Angeles, CA) procedure is a solid-phase radioimmunoassay in which $^{125}$I-progesterone competes for a fixed time with progesterone in the experimental sample for antibody sites. Since the antibody is immobilized to the wall of a polypropylene tube, decanting the supernatant is sufficient to terminate competition and to isolate the antibody-bound fraction of the radiolabeled progesterone. Counting the tube in a gamma counter yields a number, which converts by way of a calibration curve to a measure of the progesterone present in the sample.

Materials Supplied in Kit

Progesterone antibody-coated tubes

Iodinated progesterone ($^{125}$I-progesterone) supplied in liquid form.

Progesterone Standards (0, 0.1, 0.5, 2, 10, 20 and 40 ng progesterone/ml, equivalent to 0, 10, 50, 200, 1000, 2000 and 4000 pg progesterone/100 μl/tube).

Materials Prepared in Lab

Plain uncoated 12 x 75 mm polypropylene tubes- for use as NSB tubes.

Plasma from an ovariectomized cow.
Standard solutions of progesterone prepared in ovariectomized plasma via a serial dilution as follows from a stock solution of progesterone (Sigma Chemical Co.) in benzene (50 μg/ml):

X: 1000 pg/100 μl = 1μl stock + 5 ml OVX cow plasma
A: 500 pg/100 μl = 1 ml X + 1 ml OVX cow plasma
B: 250 pg/100 μl = 1 ml A + 1 ml -"-
C: 125 pg/100 μl = 1 ml B + 1 ml -"-
D: 62.5 pg/100 μl = 1 ml C + 1 ml -"-
E: 31.25 pg/100 μl = 1 ml D + 1 ml -"-

100 μl plasma/tube was used in the assay.

Assay Procedure

1. Label four uncoated 12 x 75 mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate.

2. Label tubes coated with progesterone antibody for Zero binding (Bo), the standard curve (31.25, 62.5, 125, 250, 500, 1000 pg/100 μl/tube in OVX cow plasma, the samples and reference controls in duplicate.

3. Pipet 100 μl OVX cow plasma into the NSB and Bo tubes.

4. Pipet 100 μl of the standards and samples into appropriate tubes. If sample is less than 100 μl, adjust to 100 μl with OVX plasma.

5. Add 1.0 ml of ^{125}I-progesterone to every tube and vortex. No more than 10 min should elapse between the first and last tubes during addition of the tracer.
6. Set the T tubes aside for counting at end of assay; they require no further processing.

7. Incubate all tubes at room temperature for 3 h.

8. Decant contents of all tubes (except the T tubes) and allow to drain for 2-3 min. Strike the tubes sharply on absorbent paper to shake off all residual droplets.

9. Count all tubes for 1 min in a gamma counter.

**Progesterone RIA Protocol**

<table>
<thead>
<tr>
<th>Tube</th>
<th>OVX (µl)</th>
<th>Standard/ Sample (µl)</th>
<th>$^{125}$I-P&lt;sub&gt;4&lt;/sub&gt; (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NSB</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Zero</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Standards</td>
<td>0</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Sample</td>
<td>0</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Reference</td>
<td>0</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>
Calculation of Results

Progesterone concentrations are calculated from a logit-log representation of the calibration curve. Calculate the average NSB-corrected counts per minute for each pair of tubes, and then determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%:

\[
\text{Percent Bound} = \frac{\text{Net Counts}}{\text{Net MB Counts}} \times 100
\]

Validation of Assay

The progesterone kit originally designed for human serum was validated for use with cow plasma, as described in chapter 3.
APPENDIX 3
CULTURE AND RADIOLABELLING OF LUTEAL TISSUE

Time Course Studies of Incorporation of Radiolabel

Introduction

A time course study was carried out with CL from 3 animals to determine the time of incubation required to obtain optimum incorporation of radiolabel into proteins released into culture medium. Five Petri dishes of luteal tissue (500 mg/dish) were prepared from each corpus luteum. Each of the five was given the same treatment except for the length of incubation.

Procedure

Pre-incubation of Luteal Tissue without Radiolabel

1. Prepare five culture dishes of luteal slices (500 mg/ Petri dish) for each CL.
2. Incubate luteal slices (500 mg/dish) in 15 ml leucine-deficient incomplete MEM without radiolabel for 2 h at 37 °C in a gas chamber mixing equal volumes of 100% N₂ and a 90:10, O₂:CO₂ mixture, and maintain the temperature at 37 °C by using a Fisher Isoterm incubator model 255D (Springfield, NJ).
3. After pre-incubation, take Petri dishes out of the incubator, and discard
Incubation of Luteal Tissue with $^3$H-leucine

4. Add 15 ml of pre-warmed incomplete MEM (15 ml/Petri dish) to tissue slices in each dish.

5. Add 50 $\mu$Ci of L-[4,5-$^3$H-leucine (160 Ci/mmol) to each Petri dish and swirl gently to mix.

6. Pipet 10 $\mu$l of medium from each dish into a scintillation vial, add 3 ml scintillation cocktail and count to obtain pre-incubation counts.

7. Then place Petri dishes in the gas chamber and incubate under same atmospheric conditions.

8. Take out one of the five Petri dishes after 6, 12, 18, 24 and 30 h, respectively of incubation.

9. Transfer tissue and medium from Petri dish into a 15 ml centrifuge tube.

10. Centrifuge at 2000 x g for 20 min to separate luteal-conditioned medium (LCM) from the tissue.

11. Transfer the supernatant (conditioned-medium) from each Petri dish into a separate dialysis tubing (Spectra/Por$^R$ 3 membrane, molecular weight cutoff = 3500) (Spectrum Medical Industries Inc., Houston, TX).

12. Dialyze medium against 2 changes (24 h each) of 4 l Tris-HCl buffer (10 mM pH 8.2) at 4 °C, and then against 2 changes (24 h each) of 4 l deionized water.

13. Following dialysis, transfer the retentate (dialyzed luteal-conditioned
medium) to a 50 ml centrifuge tube, and measure total volume of retentate. Volume should be 15 ml or close to that.

14. Pipet 10 µl of each retentate in triplicate into a scintillation vial and count by scintillation spectroscopy to obtain post-dialysis counts for each incubation period (see appendix 4).

15. Store the retentates at -20 °C.

**Radiolabeling with ³H-leucine**

Results of the time course study showed that the 24 h-incubation time was optimal for incorporation of radiolabel into proteins. Thus 24 h was used for subsequent incubations.

**Procedure**

1. Prepare two Petri dishes of luteal slices (500 mg/dish) from each CL.

2. Carry out pre-incubation without radiolabel for 2 h as described earlier.

3. Discard spent medium and carry out incubation with radiolabel for 24 h as described above.

4. Before incubation, take 10 µl of medium (in triplicate) and count by scintillation spectroscopy (pre-incubation counts).

5. Following incubation and dialysis, take 10 µl of retentate and count by scintillation spectroscopy (post-dialysis counts).

6. Measure volume of dialyzed LCM (retentate) from each Petri dish and adjust to 15 ml with deionized water if < 15 ml.

7. Store retentates at -20 °C until further analysis.
Radiolabeling with $^3$H-glucosamine and $^{35}$S-methionine

In addition to radiolabelling with $^3$H-leucine, luteal slices (500 mg/dish) were also incubated with $^3$H-glucosamine (50 µCi/15 ml, n = 1 cow) or $^{35}$S-methionine (50 µCi/15 ml, n = 1 cow) following the same protocol, to determine if newly synthesized and secreted proteins were glycosylated and/or contained methionine. Liver tissue was also incubated in the same manner with $^3$H-leucine to evaluate the tissue specificity in protein synthesis and secretion. Retentates from these incubations were also stored at -20 °C until further analysis.

Lyophilization of Retentates

1. Freeze-dry each frozen retentate at -50 °C under vacuum.
2. Weigh and record weight of each lyophilized sample.
3. Store samples at -20 °C until further analyses.
APPENDIX 4
DETERMINATION OF INCORPORATION OF RADIOLABEL INTO NEWLY-
SYNTHESIZED PROTEINS

A. Percent Incorporation

1. Add 3 ml scintillation cocktail to 10 μl of culture medium (in triplicate) prior to addition of \(^3\)H-leucine, and measure radioactivity (pre-incubation counts) by scintillation spectroscopy (2200CA TRI-CARB scintillation analyzer, Packard Instrument Company).

2. After dialysis, take 10 μl of retentate (in triplicate) and count by scintillation spectroscopy (post-dialysis counts).

3. Calculate Percent Incorporation of \(^3\)H-leucine as post-dialysis counts divided by pre-incubation counts x 100%, for each sample.

B. Measurement of TCA-Precipitable Radioactivity

1. Cut one-inch squares of Whatman 3MM filter paper (Whatman Ltd., Maidstone, England) and number using a pencil.

   Squares should be prepared in duplicate for each sample.

2. Soak numbered squares in 20% TCA and let air dry completely (could use heat lamp to promote drying).

3. Prepare a series of 500 ml plastic beakers (reservoir beakers) containing 20% TCA, 5% TCA, 5% TCA, 95% ethanol, 95% ethanol, respectively.
4. Cut holes into the bottom of a beaker small enough to fit into the reservoir beakers.

5. Place a radioactive tape on all equipment used.

6. Bring out post-dialysis retentates from the -20 °C freezer to warm to room temperature.

7. After the squares are completely dry, spot 50 μl of each retentate onto each square. Dispense the samples slowly to avoid loss. Spot each sample in duplicate.

8. Place the spotted squares on a tray double-lined with bench paper, and allow to air dry completely. It would take at least 30 min.

9. When completely air dry, pick up the squares with a pair of tweezers and place in the beaker with holes at the bottom.

10. Soak the squares successively in 20% TCA, 5% TCA, 5% TCA, 95% ethanol and 95% ethanol for 10, 20, 10, 10, and 5 min, respectively, by fitting the beaker with holes into each reservoir beaker.

11. Retrieve the squares and place on a tray for 1 h to completely air dry.

12. Pick up the dried squares using tweezers and place in scintillation vials.

13. Add 3 ml scintillation cocktail to each vial and count radioactivity on a beta counter.

14. Obtain and record counts (dpm and cpm) in duplicate for each sample.
First-dimension: Isoelectric Focusing (IEF) in Tube Gels was carried out according to method of Laemmli (1970).

Preparation of Glassware and Reagents for IEF

Glass Tubes:

1. Soak IEF glass tubes (inner diameter 2.5 mm) in concentrated nitric acid for at least 24 h.
2. Remove the glass tubes from the acid and soak in deionized water to rinse off excess acid.
3. Soak tubes in 0.2% (w/v) potassium hydroxide (2 g KOH/l in 95% ethanol) for at least 2 h.
4. Rinse tubes extensively with deionized water and place in a beaker lined with paper napkins to drain. Ensure complete dryness by placing the beaker in a drying oven overnight.
5. Prior to use, seal one end of each tube with parafilm (at least three layers).
6. Mark each tube at 12 cm from the sealed end to maintain same length in
tube gels.

7. Place the tubes in the gel casting stand with sealed ends down.

**Pouring of Tube Gels:**

1. Fill the IEF tubes to the mark with the IEF gel solution using a 10 cc syringe and a 20 gauge 5 inch-long needle with tubing at the tip.
2. Overlay tube gels with 20 µl 8 M urea.
3. Overlay the urea layer with 30 µl deionized water.
4. Cover the tubes with clear plastic and leave at room temperature for gels to polymerize.

**Isoelectric Focusing**

**IEF Pre-run:**

1. After the gels polymerize, carefully aspirate the gel overlays using a Pasteur pipet and a long pipet tip.
2. Set up the IEF gel apparatus. Fill the lower reservoir with anolyte solution. Stand the tube holder (upper reservoir) inside the lower tank to separate the upper and lower reservoirs.
3. Take each gel tube out of the casting stand, and remove the parafilm from the sealed end.
4. Dip that end into glycerol (to eliminate air and facilitate insertion of tube through bushings in the tube holder), and insert tubes through the holes into the lower reservoir, making sure the top of each gel is visible over the
bushings.

5. Fill the gel tubes to the brim with catholyte solution. Then pour more catholyte solution into the upper reservoir so that all the tubes are submerged.

6. Connect the gel apparatus to a cooling apparatus pre-set at 20 °C, and a power supply unit set at constant voltage.

7. Carry out an isoelectric focusing pre-run for 15 min at 200 V, then for 30 min at 300 V and for 30 min at 400 V.

8. In the meantime, prepare the samples for loading.

**Sample Preparation>Loading:**

1. Take lyophilized retentates out of the freezer.

2. Weigh the amount of each sample equivalent to 500,000 cpm.

3. Dissolve each sample in 200 µl IEF sample buffer. Spin solution in microfuge for 2 min to precipitate any insoluble material.

4. Pipet 10 µl of supernatant into a scintillation vial, add 3 ml scintillation cocktail and count radioactivity.

5. Based on the counts for the 10 µl aliquot, calculate the volume of sample equivalent to 100,000 cpm for each sample.

6. After the pre-run, turn off power supply and cooling units.

7. Siphon off the catholyte solution in the upper tank.

8. Aspirate (using autopipet and long pipet tip) the layer of catholyte overlay on the gels.
9. Overlay each tube gel with 10 μl of IEF sample buffer.

10. Then load the sample (100,000 cpm) on top of the tube gel.

11. Overlay the samples with catholyte solution to the brim of the glass tubes.

12. For every IEF run, load one tube gel with only sample buffer, to serve as the pH gradient of the run.

13. After loading the samples, fill the upper reservoir with fresh catholyte solution.

14. Reconnect the cooling unit and power supply.

15. Set the voltage at 400 V and carry out isoelectric focusing for a minimum of 8000 Vh.

16. After electrofocusing, remove IEF tubes from the apparatus, and extrude the IEF gel by attaching a tube at the end of a syringe filled with deionized water, to one end of the IEF tube and pushing the plunger.

17. Nick the acidic end (bottom) of the IEF gel for orientation.

18. Either use the IEF tube gels immediately for the second-dimension slab gel electrophoresis or store tube gels individually in 18 x 150 mm glass tubes at -20 °C until further analysis.

Determination of pH Gradient:

1. Extrude the IEF tube gel containing no sample (control gel) from the glass tube after electrofocusing.

2. Cut the control gel into 0.5 cm pieces sequentially from the acidic to the basic end.
3. Place each piece in a 1.5 ml bullet tube vial containing 600 μl of deionized water.

4. Place the vials on a rocker platform at room temperature overnight, to allow sippage of ampholine out of gels into the water.

5. Measure pH of the solution in each vial, beginning at the acidic end.

Second Dimension Electrophoresis

Preparation of Acrylamide Separating Gel (10% T, 2.7% C<sub>bis</sub>)

In a 125 ml side arm vacuum flask with a small magnetic stir bar, the separating gel solution was prepared by mixing the following:

- 20 ml Acrylamide Stock Monomer solution (30% T, 2.7% C<sub>bis</sub>)
- 15 ml 4X Running Gel Buffer stock
- 0.6 ml 10% (w/v) SDS
- 24.1 ml deionized water.

Stopper the flask and deaerate the mixture by applying vacuum for several minutes.

Add 300 μl 1% (w/v) ammonium persulfate, mix, and add 20 μl TEMED to 60 ml.

Swirl the flask gently to mix, avoid generating air bubbles.
Pouring of Separating Gel Mixture:

1. Pour the separating gel mixture into the sandwich to the mark using a 60 ml syringe.
2. Overlay the gels with 3 ml deionized water and allow to polymerize (about 1 h).
3. Pour off the overlay by tilting the casting stand.
4. Overlay (equilibrate) gels with 3 ml Running Gel Overlay Solution for at least 2 h.

Preparation of the Stacking Gel (4% T, 2.7% C_{bis})

In a 125 ml side arm flask containing a magnetic stir bar add:

- 2.66 ml Acrylamide Stock solution (30% T, 2.7% C_{bis})
- 5.0 ml 4X Stacking Gel Buffer
- 0.2 ml 10% (w/v) SDS
- 12.2 ml deionized water.

Deaerate the solution, then add

- 100 μl 1% (w/v) ammonium persulfate, and
- 10 μl TEMED to 20 ml.

Swirl to mix.

Pouring of Stacking Gel Mixture

After equilibration, pour off the Running Gel Overlay.

1. Clamp the stacking gel caster on top of the separating gel assembly.
2. Rinse the surface of the polymerized separating gel with 1-2 ml of
stacking gel solution.

3. Rock the casting stand manually to wash the surface, and pour off the liquid.

4. Overlay the polymerized separating gel with the remaining stacking gel solution until latter is visible above the grooves of the casting gel stand, using a Pasteur pipet and pipet tip.

5. Overlay the stacking gel with deionized water and allowed to polymerize (45 min).

2-D Electrophoresis:

Following isoelectric focusing,

1. Equilibrate the tube gels (100,000 cpm/IEF gel) in gel equilibration buffer (0.0625 M Tris, 5% (w/v) SDS, 10% (v/v) glycerol) for 15 min.

2. Lay the equilibrated IEF gel in the grooves. Take note of the orientation (acidic/basic ends).

3. Place a molecular weight marker worm to the left of the IEF gel.

4. Dispense hot 0.1% (w/v) agarose into the groove with a transfer pipet. This will connect the IEF gel to the stacking gel when the agar sets.

5. Place the assembly in the lower electrophoresis tank, fill the upper reservoir with tank buffer, place the lid, and connect the assembly to the power supply unit.

6. Carry out electrophoresis at a constant current of 13 mA/gel until the dye front reaches the end of the glass plates.
7. Remove gels from the apparatus, and detach the stacking from the separating gel.

8. Nick the acidic end of the separating gel for orientation.

9. Stain separating gel with 0.1% (w/v) Coomassie Blue for at least 2 h on a rocker platform.

10. Destain gels in destaining solution (see below) overnight on a rocker platform.

**Fluorography and Densitometry**

1. Soak destained gels in deionized water for at least 30 min.

2. Soak gels in 1 M sodium salicylate for 30 min.

3. Place gels on filter paper (8 in x 10 in) pre-wet with deionized water.


5. Mark positions of the molecular weight markers on the filter paper with pencil. Also write the sample number.

6. Expose dried gels to x-ray film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY) for 4 wk at -80 °C.

7. Identify radiolabeled proteins by matching spots on the fluorographs with those on the Coomassie-stained gel.

8. Determine the intensity of the spots by densitometric scanning (E-C Apparatus Corporation, St Petersburg, FL).

9. Measure the area under the curve for each spot after scanning with a
planimeter (Model 1250, Numonics Corporation, Lansdale, PA).

Second-Dimension:

Preparation of Glassware, Equipment and Reagents:

1. Soak glass plates (8 cm x 15 cm) in concentrated nitric acid for at least 24 h.
2. Rinse the plates extensively with deionized water, wipe dry with kinwipes, and wipe with 95% ethanol.
3. Also wipe the spacers (1.25 mm in thickness), rubber bushings, clamps and the gel caster with 95% ethanol.
4. Pair the glass plates, separated by 2 spacers to make a sandwich.
5. Clamp the sandwich and mount it on the gel caster lined with rubber bushings which block the bottom end of the sandwich, to prevent leakage of the gel solution before it polymerizes.
6. Mark the glass plates 3 cm from the top, to serve as the top of the separating gel.

Solutions

Acrylamide Monomer Stock Solution (18% T, 5.4% C_{bis}):

- 43.8 ml of 40% (w/v) acrylamide
- 50.0 ml of 2% (w/v) C_{bis} acrylamide
- 6.2 ml deionized water to 100 ml.

Store at 4 °C in a dark bottle.
IEF Tube Gel Solution (4% T, 5.4% C\textsubscript{bis})

In a 125 ml flask with side arm, add:

- 3.24 ml acrylamide monomer stock (18% T, 5.4% bis)
- 8.25 g urea
- 1.17 ml deionized water
- 3.0 ml 10% NP-40
- 0.75 ml ampholine (pH range 3.5-10.0)

Swirl to dissolve urea.

Degas the solution, then add

- 15 µl of 10% (w/v) ammonium persulfate

Mix by swirling, and add

- 11 µl TEMED. Mix by swirling.

IEF Gel Sample Buffer (9 M urea, 2% NP-40, 2% ampholine)

Into a 50 ml graduated centrifuge tube, add:

- 2.85 g urea
- 1.0 ml 10% (v/v) NP-40
- 250 µl ampholine (pH range 3.5-10.0). Swirl to mix.

Deionized water to 5 ml.

Aliquot (500 µl) into 1.5 ml bullet tubes and store at -20 °C.

IEF Anolyte Stock Solution (1 M Phosphoric Acid):

- 17 ml 85% (v/v) H\textsubscript{3}PO\textsubscript{4}

Deionized water to 250 ml.
IEF Anolyte Working Solution

40 ml of anolyte stock solution
Deionized water to 4 l. Prepare just before use.

IEF Catholyte Stock Solution (1 M Sodium Hydroxide)

10 g sodium hydroxide
Deionized water to 250 ml.

IEF Catholyte Working Solution

36 ml catholyte stock
Dilute to 1.8 l with deionized water.
Prepare prior to use in a 2-liter flask with side arm. Degas extensively before use.

Gel Overlay Solution (8 M urea)

4.8048 g urea
Deionized water to 10 ml.
Aliquot (100 µl) in 500 µl bullet tubes and store at -20 °C.

Monomer Stock Solution (30% T, 2.7% C_{bis})

58.4 g acrylamide
1.6 g bis acrylamide
Deionized water 200 ml.
Store at 4 °C in a dark bottle.
**4X Running Gel Buffer** (1.5 M Tris-Cl, pH 8.8)

36.3 g Tris (pH 8.8)
Deionized water to 200 ml.
Adjust pH to 8.8 with 1 M HCl.

**4X Stacking Gel Buffer** (0.5 M Tris-Cl, pH 6.8)

3.0 g Tris
Deionized water to 50 ml
Adjust the pH to 6.8 with 1 M HCl.

**Sodium Dodecyl Sulfate** (10% SDS)

10 g SDS
Deionized water to 100 ml.

**Initiator** (10% ammonium persulphate)

100 mg ammonium persulfate
Deionized water to 1 ml.
Prepare just before use.

**Running Gel Overlay** (0.375 M Tris-Cl, 0.1% SDS, pH 8.8)

25 ml of 4X Running Gel Buffer
1.0 ml 10% (w/v) SDS
Deionized water to 100 ml.

**2X Treatment Buffer** (0.125 M Tris-Cl, 4% SDS, 20% glycerol, pH 6.8)

4X stacking gel buffer (2.5 ml)
10% (w/v) SDS (4.0 ml)
Glycerol (1.0 ml)

Deionized water 10 ml.

Aliquot (1 ml) into 1.5 ml bullet tubes and store at -20 °C.

**Tank Buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3)**

2.5 g Tris

72 g glycine

5 g SDS

Deionized water to a final volume of 5 l. It is not necessary to check the pH of this solution.

**Stain (0.1% Coomassie Blue R-250, 40% ethanol, 7% acetic acid)**

1 g Coomassie Blue

400 ml 95% (v/v) ethanol (stir to dissolve)

70 ml acetic acid

Deionized water to a final volume of 1 liter.

Filter before use.

**Destaining Solution I (50% ethanol, 10% acetic acid)**

Ethanol (500 ml)

Acetic acid (100 ml)

Deionized water to 1 liter.

**Destaining Solution II (7% acetic acid, 5% ethanol)**

Acetic acid (700 ml)

Ethanol (500 ml)
Deionized water to 10 liters.

**Bromophenol Blue (0.5% in 10% ethanol)**

50 mg Bromophenol Blue

Ethanol to 10 ml.
APPENDIX 6  
ELECTRO-BLOTTING OF PROTEINS TO MEMBRANE

Introduction

Proteins of interest were blotted to polyvinylidene difluoride (PVDF) membranes according to procedure by Towbin et al. (1979).

Procedure

1. Carry out two-dimensional SDS polyacrylamide gel electrophoresis as previously described.

2. Place the gel (equilibrate) in blotting buffer (morpholino-ethane sulfonic acid (MES, pH 6.0) for 20-30 min.

3. Pre-wet a piece of PVDF membrane (same size as gel) in a small volume of 100% methanol for 1-2 sec.

4. Rinse PVDF membrane in deionized water to remove the excess methanol.

5. Place membrane in the blotting buffer to equilibrate for 10-20 min prior to use.

6. Equilibrate Whatman™ type 3MM chromatography paper (Whatman Ltd., Maidstone, England) and support foam sponges in blotting buffer prior to use.

7. Arrange the blotting assembly in a sandwich configuration (-) fibre
sponge-2 Whatman paper-gel-PVDF membrane-2 Whatman paper-fibre sponge (+), and clamp in sandwich holder.

8. Place the sandwich assembly with the membrane facing the anode in the Bio-Rad Model TRANS-BLOT™ CELL blotting unit (Bio-Rad Laboratories, Richmond, CA).

9. Place assembly in a cold room (4 °C) and carry out electrotransfer at a constant voltage (20 V) for 16 h with stirring.

**Staining and destaining of blots**

1. Disassemble the blotting unit and retrieve the PVDF membrane.

2. Place blots in staining solution for 2-5 min.

3. Destain blots in destaining solution (50% (v/v) ethanol, 10% (v/v) acetic acid) for 3-5 min.

4. Wash blots extensively with many changes of distilled water.

5. Place blots between Whatman No. 3 paper to completely air dry.

6. Place dried blots in plastic bags, wrap the bags in aluminium foil, and store at -20 °C until further analysis.

**Solutions**

**Transfer Buffer (10 mM MES Buffer, pH 6.0, 20% Methanol)**

1.95 g MES (pH 6.0)

200 ml methanol

Make up to 1 liter with distilled water.
Staining Solution (0.1% (w/v) Coomassie Blue R-250, 50% (v/v) ethanol, 7% (v/v) acetic acid)

1 g Coomassie Brilliant Blue R-250
500 ml 100% ethanol
70 ml glacial acetic acid
Make up to 1 liter with distilled water.

Destaining Solution (50% ethanol, 10% acetic acid)
500 ml 100% ethanol
100 ml glacial acetic acid
Make up to 1 liter with distilled water.
APPENDIX 7
DETERMINATION OF TOTAL PROTEIN IN RETENTATE BY METHOD OF LOWRY

Procedure
1. Add samples and standards to appropriate tubes (200 µl/tube). If sample volume is less than 200 µl, make up to that volume with double-distilled water.
2. Add 3.0 ml of solution I (see below) to each tube, mix briefly and let stand at room temperature for 15 min.
3. Add 0.3 ml of solution II (see below) to each test tube, mix immediately and let stand for 35 min at room temperature.
4. Turn on spectrophotometer (use red filter).
5. Read optical density at 750 nm under UV light.
6. Plot a standard curve of optical density versus concentration. Read off concentration of unknown samples from the standard curve.

Solutions
Protein Standards
The standards used were prepared fresh by diluting bovine serum albumin (BSA) in water to obtain: 0 (blank), 5, 10, 20, 50, 100, 200 µg BSA.
Stock Solutions

Solution A

2% Na$_2$CO$_3$, 0.1 N NaOH
20 g Na$_2$CO$_3$
4.0 g NaOH
Dissolve in double-distilled water to 1 l.

Solution B

1% CuSO$_4$.5H$_2$O
1 g dissolved to 100 ml with double-distilled water

Solution C

2% Sodium Potassium Tartrate
2 g dissolved to 100 ml with water.

Working Solutions

Mix equal volumes of solutions B and C and let stand at room temperature for 10 min.

Solution I

Add 50 ml of solution A to 1 ml of the mixture of solutions B and C, mix immediately and let stand for 15 min.

Solution II

Dilute Folin Reagent (2 N, Sigma Chemical Co.) 1:1 with distilled water to obtain 1 N Folin Reagent.
APPENDIX 8
MEASUREMENT OF APO A-1 MRNA

Preparation of Apo A-1 Plasmid DNA

Apo A-1 plasmid DNA in E coli was purchased from ATCC. The plasmid DNA was in lyophilized form with the following specifications:

- freeze-dried E coli containing plasmid
- medium: 1273, LB plus tetracycline
- insert contains Apo A-1 cloned from adult human liver
- detects sequence: human Apo A-1, 11 q23-q24 - insert size: 0.6 KB - total size: 4.4 KB
- source of insert DNA: cDNA
- name of vector: pKT218
- insert site: Pst1
- insert ends: Pst1
- excise with: Pst1
- markers: tet\textsuperscript{R}
- sequence corresponds to amino acids 94-243, contains 3' untranslated sequence plus poly (A) tail.
Procedure

A. **Reconstitution**

1. Add 200 µl sterile TE buffer (pH 7.4 or pH 8) to the vial containing the lyophilized plasmid DNA (stock). Store the stock at -20 °C for long-term, or at -80 °C for very long-term storage.

B. **Cultivation of Plasmid DNA**

1. Take out 50 µl transformed bacteria competent cells (JM 109, Promega Corporation, Madison, WI) from the stock (usually stored at -80 °C) to thaw on ice. Return stock transformed cells to -80 °C immediately after use.

2. Place 5 µl of reconstituted plasmid DNA in a snap cap tube and placed on ice for 30 min.

3. In the meantime, take out LB-tet\(^R\) plate from -20 °C and place in incubator upside down at 37 °C to pre-warm. Label the plates.

4. Heat shock the mixture in 2 by placing in a water bath at 42 °C for 90 sec.

5. Place mixture on ice for 2 min.

6. Add 100 µl SOC medium (see below) and place in an incubator with a shaker at 37 °C for 1 h.

7. Take out mixture from incubator and transfer to a laminar flow bacteria hood.

8. Turn on the blower and light in the hood.

9. Then take out the tet\(^R\) plate from the incubator to the hood.
10. Carefully open the plate under the hood, and in a single motion dump the DNA mixture from # 7 onto the plate.

11. Spread out the DNA-competent cell mixture on the plate using the sides of a sterile autopipet tip (large).

12. Leave the plate half-covered and allow to air dry for about 5 min in the hood.

13. Take plate out of hood and place in the incubator (37 °C) upside down, overnight. Turn off blower and light in hood.

Isolation of plasmid DNA

14. Next day, take out plate from incubator and examine for presence of colonies.

15. Seal off ends of the plates with parafilm and store at 4 °C until ready for use.

16. With use of an autopipet, pick one colony from the plate and add to 200 ml sterile LB medium (see below) in a sterilized 500 ml culture bottle. Add 75 µl of tetracycline stock (10 mg tetracycline in 50% ethanol, 50% water) for every 50 ml of LB medium i.e. add 300 µl tetracycline stock for 200 ml LB medium. For ampicillin resistant clones, use 20 mg ampicillin (Na salt) for 200 ml LB medium (weigh 20 mg ampicillin and add directly to LB prior to use.

17. Cover the bottle loosely, place in a styrofoam box padded with paper towels, and place assembly in an incubator at 37 °C overnight with
shaking.

18. Next day, take out 1 ml of the overnight culture of E coli containing plasmid DNA and put in a snap cap tube. Add 1 ml freeze broth to tube and place in incubator (37 °C) on a shaker overnight to grow. Freeze broth is usually stored at -80°C as a stock in case it is needed in future.

19. Pour out the rest (199 ml) of the overnight culture in # 18 into a wide-mouthed 250 ml plastic bottle with a screw cap.

20. Centrifuge mixture at 4000 x g (Sorvall model RC-5B, HS-4 rotor head) for 15 min at 4 °C.

21. Decant supernatant back into the 500 ml culture bottle that was used in step # 16, add bleach and let sit for 10 min before pouring down the drain. Save the pellet (containing the plasmid DNA) for further processing.

22. From this step, the plasmid DNA was prepared using QIAGEN-tip 100 plasmid kit (Qiagen Inc., Chatsworth, CA) midi protocol as follows:

23. Add 4 ml of buffer P1 (resuspension buffer) to the pellet obtained in step # 21. Use a sterile Pasteur pipet to squirt the P1 buffer over the pellet to obtain a suspension.

24. Transfer the suspension to a smaller plastic tube with screw cap, using a sterile transfer pipet.

25. Add 4 ml buffer P2 (lysis buffer), mix by vortexing and incubate at room temperature for 5 min.

26. Add 4 ml chilled buffer P3 (neutralization buffer), mix, and incubate on ice
for 15 min.

27. Centrifuge at 4 °C for 15 min at 47,800 x g (Sorvall model RC-5B, ss 34 rotor head).

28. Equilibrate a QIAGEN-tip 100 column with 4 ml of buffer QBT (equilibration buffer). Let the column suspend on holders over the 250 ml plastic bottle used in step # 19.

29. Apply the supernatant from step # 27 onto the QIAGEN-tip 100 column.

30. Wash the QIAGEN-tip 100 twice with 10 ml of buffer QC (wash buffer).
    Discard eluate.

31. Transfer and place the column and holder over a sterile hard plastic tube.

32. Elute the DNA bound to the column with 5 ml of buffer QF (elution buffer).
    Save the eluate.

33. Add 3.5 ml (0.7 volumes of reconstituted plasmid DNA started with) isopropanol to precipitate the DNA.

34. Centrifuge at 47,800 x g for 45 min (Sorvall ss 34 rotor head) at 4 °C.

35. Discard the supernatant and carefully look for an opaque spot around the curved region of glass tube. That opaque spot is the DNA pellet.

36. Add 5 ml of cold 70% ethanol (stored at -20 °C) to the pellet to wash the DNA, centrifuge at 47,800 x g (Sorvall ss 34 rotor head) for 10 min.

37. Place the tube face down on paper towels to drain and air dry for 5 min.

38. Redissolve DNA pellet in 50 μl of Tris-EDTA buffer (see below). This is the purified plasmid DNA stock. Use some of the plasmid DNA to
determine concentration and size of the plasmid.

39. Store the rest of the plasmid DNA at 4°C until further analysis.

**Measurement of concentration of Apo A-1 plasmid DNA**

1. Pipet 995 µl of sterile water into a 12 x 75 mm test tube.
2. Add 5 µl of purified plasmid DNA to the tube. Vortex to mix.
3. Measure optical density of mixture at 260 nm on a spectrophotometer.
4. Calculate DNA concentration.

**Digestion (lysis) of Apo A-1 plasmid DNA**

The plasmid DNA is digested in order to release the Apo A-1 DNA insert from the plasmid.

**Procedure**

1. Pipet 2 µl of purified plasmid DNA into a 0.5 ml bullet tube.
2. Add 3 µl Pst1 buffer, 22 µl sterile water, and 3 µl Pst1 enzyme, to obtain a final volume of 30 µl. Pipet up and down to mix.
3. Incubate mixture in a water bath at 37 °C overnight.
4. Separate plasmid digest by electrophoresis.

**Separation of Plasmid Digest by Agarose Gel Electrophoresis**

**Agarose gel Preparation**

1. Dissolve 0.6 g agarose in 50 ml TAE buffer (see below) (1.2 % gel).
2. Heat to a boil in a microwave until agarose is completely dissolved.
3. Add ethidium bromide (4 µl) to the gel mixture, mix, allow gel to cool, and then pour onto a mini gel casting tray with comb. Pour from end of plate
away from the comb. Avoid air bubbles in the gel.


5. When set, place the gel tray in the tank containing 1X TAE buffer.

6. Gently pull out the comb, take off the rubber bungs and pour more 1X TAE buffer to cover gel.

Loading of Samples and Electrophoresis

Preparation of Lamda Markers

Pipet 20 µl TE buffer

4 µl lamda markers

5 µl DNA loading dye, in a bullet tube.

Place mixture in a water bath (62 °C) for 2-5 min.

Preparation of Samples

Add 5 µl of DNA loading dye to sample digest.

Mix well using autopipet.

Electrophoresis

1. Load lamda markers in first lane, and plasmid digest on subsequent lanes.

2. Cover the electrophoresis tank, and set voltage between 81-92 V (E-C Apparatus Corporation, St. Petersburg, FL) with voltage setting range set at 'low'.
3. Run electrophoresis until dye front is about midway in gel. The wells should be at the black terminal of the tank cover.

4. Turn off power, take out gel and examine under UV light.

5. Compare migration of sample with that of DNA markers to determine if insert is of the right size. Take a picture of the gel.

6. If insert is of the right size, carry out a digestion of plasmid DNA on a larger scale, separate digests on agarose gel as before, and transfer inserts by electroblotting from the gel to DEAE paper.

Solutions

**SOC Medium**

Bacto tryptone (2%)

Bacto yeast extract (0.5%)

Sodium chloride (10 mM)

Potassium chloride (2.5 mM)

Magnesium chloride (10 mM)

Magnesium sulfate (10 mM)

Glucose (20 mM)

Combine tryptone, yeast extract, NaCl, and KCl in sterile water and autoclave 30-40 min.

Make a 2 M stock of Mg$^{2+}$, comprised of 1 M MgCl$_2$ and 1 M MgSO$_4$. Filter sterilize (22 µ membrane).

Prepare a 2 M stock of glucose similarly and store at -20°C.
Prior to use, combine the media with Mg and glucose and filter sterilize.

**Freeze Broth**

22 g Glycerol
5 g Tryptone
2.5 g Yeast Extract
2.5 g NaCl
3.15 g K$_2$HPO$_4$
0.9 g KH$_2$PO$_4$
0.23 g Sodium citrate
0.45 g (NH$_4$)$_2$SO$_4$

Dilute to 500 ml, and autoclave.

Add 0.25 ml 1 M MgSO$_4$ (filter-sterile).

**LB-medium, 1 liter:**

10 g Tryptone
5 g Yeast Extract
5 g NaCl
pH to 7.5 with 10 M NaOH

Dilute to 1 l with water

Autoclave and store at 4 °C

**LB Agar plates**

LB medium + 15 g Bacto-agar

Autoclave
When cool, add 100 mg ampicillin (or tetracycline), plus 1 ml X-Gal (20 mg/ml in NN dimethyl formamide).

**TE buffer, pH 8.0**

10 mM Tris-HCl

1 mM EDTA
APPENDIX 9
MEASUREMENT OF APO E MRNA

Preparation of Apo E Plasmid DNA

Apo E plasmid DNA in E coli was purchased from ATCC. The plasmid DNA was in lyophilized form with the following specifications:

- freeze-dried E coli containing plasmid
- medium: 1273, LB plus tetracycline
- insert contains Apo E cloned from adult human liver
- detects sequence: human Apo E, 11 q23-q24 - insert size: 0.6 KB- total size: 4.4 KB
- source of insert DNA: cDNA
- name of vector: pKT218
- insert site (s): Pst1
- insert size: 0.9 kb
- excise with: Pst1
- markers: tetR
- sequence encodes amino acids 81-299, and contains the 158 bp 3’ untranslated region and 44 bp poly (A). The insert has 5 internal Pst1 sites.
**Procedure**

**Reconstitution**

1. Add 200 μl sterile TE buffer (pH 7.4 or pH 8) to the vial containing the lyophilized Apo E plasmid DNA (stock).

2. Store the stock at -20 °C for long-term, or at -80 °C for very long-term storage.

Following reconstitution, Apo E plasmid DNA was cultivated, isolated, digested with Pst I enzyme, and separated on agarose gel, following the same protocol as with Apo A-1. Apo E plasmid DNA was stored at 4 °C until further analysis.
APPENDIX 10
MEASUREMENT OF TIMP-1 AND TIMP-2 mRNA

TIMP-1 and TIMP-2 plasmid cDNAs were a kind gift from Dr. Michael F. Smith of the University of Missouri, Columbia, MO. The plasmid DNAs were sent in the form of agar stabs in snap cap tubes.

**Ovine TIMP-1**
- 900 base pair TIMP-1 cDNA (clone 6-2)
- cloned into EcoRI/Xhol site of PBluescript SK
- linearize with BamHI and transcribe from T7 promoter to generate antisense cRNA
- linearize with KpnI and transcribe off T3 promoter to generate sense cRNA - plasmid carries ampicillin resistance gene.

**Ovine TIMP-2**
- 438 base pair TIMP-2 cDNA (MMI)
- cloned into BamHI/EcoRI site of PBluescript SK
- linearize with XbaI and transcribe off T7 promoter to generate antisense cRNA
- linearize with Kpn and transcribe off T3 promoter to generate sense cRNA- plasmid carries ampicillin resistance gene.
Growth of Bacterial Cultures

1. Add 1 ml of LB-amp (ampicillin at 100 mg/l) to the agar stabs of TIMP-1 and TIMP-2 plasmids.
2. Incubate tubes at 37 °C overnight with shaking.
3. Next day, pipet 50 µl of overnight culture and plate onto pre-warmed LB-amp-Gal plates in a bacteria hood, as described earlier.
4. Incubate the plates at 37 °C overnight.

Purification of DNA

Isolation of Plasmid DNA

Introduction

Isolation of plasmid DNA is performed in essentially three stages. The bacterial cell wall is first weakened by the action of lysozyme, and then lysed by EDTA and a detergent at high pH. Finally, the insoluble cell debris consisting of genomic DNA and protein is precipitated with high salt and centrifuged down, leaving the plasmid DNA in solution.

Small-scale isolation of plasmid DNA: Miniprep

Procedure.

1. Pick a single bacteria colony from each LB-amp plate and suspend in 2 ml of LB-broth containing ampicillin at a final concentration of 100 µg/ml. Incubate at 37 °C overnight with vigorous shaking in an air incubator.
2. Place 1.5 ml of the overnight culture into a microcentrifuge tube and centrifuge at 12,000 x g for 1 min. The remainder of the overnight culture can be stored at 4 °C.

3. Discard the supernatant and resuspend the pellet by vortexing in 100 µl solution I (see below).

4. Incubate for 5 min at room temperature.

5. Add 200 µl of a freshly prepared solution II (see below). Mix by inversion (DO NOT VORTEX).

6. Incubate for 5 min at room temperature.

7. Add 150 µl of 3 M sodium acetate (pH 5.0). Mix by inversion.

8. Incubate for 5 min at room temperature.

9. Centrifuge at 12,000 x g for 5 min.

10. Transfer the supernatant to a fresh tube, avoiding the white precipitate.

11. Add 200 µl of water-saturated phenol and 100 µl of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 12,000 x g for 5 min.

12. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of ethanol. Mix and allow the samples to precipitate for 1 h at -80 °C.

13. Centrifuge at 12,000 x g for 15 min. Remove the supernatant and wash the pellet with 200 µl of prechilled 70% ethanol. Dry pellet
under vacuum for 5 to 10 min.


15. Add 3 µl of appropriate endonuclease buffer and 2 µl of enzyme to the DNA solution. Incubate for 16 h in a 37 °C water bath.

16. Centrifuge briefly and add 5 µl of RNase A to each tube. Incubate for 30 min at 37 °C.

17. Add 5 µl of DNA dye mix and separate the DNA fragments by electrophoresis on an agarose gel.

**Solutions**

*Solutio n I, pH 8.0:*

- 25 mM Tris-HCl, pH 8.0
- 10 mM EDTA
- 50 mM glucose

*Solutio n II:*

- 0.2 N NaOH
- 1% SDS

*3 M Sodium acetate, pH 5.2:*

Dissolve 246 g sodium acetate in 800 ml H₂O

Adjust the pH to 5.2 with glacial acetic acid

Add H₂O to 1 l

**Water-saturated phenol:**

Melt the phenol at 65 °C
Add an equal volume of \( \text{H}_2\text{O} \) and mix vigorously

Allow the phases to separate overnight at 4 °C

Large-scale isolation of plasmid DNA

Procedure

1. Inoculate 200 ml of LB-medium containing 0.1 mg/ml of ampicillin with 100 ul of stock plasmid (glycerol stock or miniprep culture).

2. Incubate overnight at 37 °C with vigorous shaking.

3. Transfer the medium to 250 ml centrifuge tubes and centrifuge for 15 min at 4000 x g (HS-4 rotor).

4. Discard the supernatant and resuspend the pellet in 3 ml of ST buffer.

   Pipet up and down to dissolve the pellet. Transfer to 50 ml polypropylene high speed tubes.

5. Add 1 ml of lysozyme (10 mg/ml in ST buffer) to each tube and vortex gently.

6. Incubate for 10 min on ice.

7. Add 2.5 ml of 0.2 M EDTA, vortex gently and let sit on ice for 5 min.

8. Add 6 ml of triton-lysis buffer and cap the tubes. Mix by inversion and let sit on ice for 8 min.

9. Invert again and let sit for another 8 min.

10. Centrifuge for 20 min at 47,800 x g (SS-34 Rotor).

11. Transfer the supernatant to a fresh tube, add 400 µl of 8 M
ammonium acetate, and vortex gently.

12. Add 12 ml of phenol and 4 ml of chloroform-isoamyl alcohol (24:1), cap and mix thoroughly by shaking.

13. Centrifuge for 5 min at 4000 x g.

14. Transfer the aqueous layer to a fresh tube and repeat steps # 12 and # 13.

15. Transfer the aqueous phase to a 50 ml disposable tube. Add 0.1 vol of 8 M ammonium acetate to each tube followed by 2 volumes of absolute ethanol.

16. Mix by inverting a few times and incubate at -80 °C for 1 h.

17. Remove the tubes from the deep freezer, let the samples thaw at RT and centrifuge for 20 min at 4000 rpm.

18. Discard the supernatant and rinse the pellet with 3 ml of 70% ethanol by centrifuging at 4000 rpm for 10 min.

19. Discard the supernatant and invert to drip-dry (approx. 10 min).

20. Resuspend the DNA pellet in 300 μl of TE buffer.

21. Add 20 μl RNase (20 mg/ml) and incubate in a 37 °C water bath for 30 min.

22. Add 150 μl of water-saturated phenol and 75 μl chloroform:isoamyl alcohol (24:1) to the tube. Mix and centrifuge at 12,000 g for 5 min.

23. Transfer the supernatant to a fresh tube and add 0.1 volume of 8 M ammonium acetate and 2.5 volume of ethanol. Allow sample to
precipitate for 1 h at -80 °C.

24. Centrifuge at 12,000 g for 15 min. Remove the supernatant and wash the pellet with 200 μl of prechilled 70% ethanol. Dry the pellet for 5 to 10 min under vacuum.

25. Resuspend the pellet (TIMP-1 or TIMP-2 plasmid DNA) in 300 μl of TE buffer and store at 4 °C.

26. Determine concentration of the plasmid DNA.

**Solutions**

**ST Buffer, pH 8.0:**

- 50 mM Tris
- 25% sucrose

**Triton lysis buffer, pH 8.0:**

- 1 ml 10% Triton-X 100
- 5 ml 1 M Tris, pH 8.0
- 62.5 ul 0.1 M EDTA, pH 8.0
- H₂O to 100 ml

**RNase:**

- 20 mg/ml of RNAse A
- boil for 15 min.

**8 M Ammonium acetate:**

- Add 61.6 g ammonium acetate
- H₂O to 100 ml
Digestion of TIMP-1 and TIMP-2 Plasmid DNA

Procedure

TIMP-1 and TIMP-2 plasmids were digested according to the following double digestion protocol:

For TIMP-1, add:

- 24 µl plasmid DNA
- 3 µl 10X high buffer (H)
- 3 µl Xhol enzyme

Mix by pipeting and place in a water bath at 37 °C overnight. Next day, spot spin and add:

- 15 µl sterile water
- 2 µl 10X high buffer (H)
- 3 µl EcoRI enzyme

Mix and place in water bath at 37 °C overnight.

Spot spin tubes and add 5 µl low RNase to all tubes.

Incubate in water bath at 37 °C for 30 min.

Spot spin plasmid digests, add 5 µl DNA loading dye to each tube and load on a 1.2 % agarose gel.

For TIMP-2, add:

- 24 µl plasmid DNA
- 3 µl 10X buffer (B)
- 3 µl BamHI enzyme

Mix by pipeting and place in a water bath at 37 °C overnight. Next day, spot spin and add:

- 15 µl sterile water
- 2 µl 10X high buffer (H)
- 3 µl EcoRI enzyme

Mix and place in water bath at 37 °C overnight.

Spot spin tubes and add 5 µl low RNase to all tubes.

Incubate in water bath at 37 °C for 30 min.

Spot spin plasmid digests, add 5 µl DNA loading dye to each tube and load on a 1.2 % agarose gel.
Electrophoresis of Nucleic Acids

Introduction

Small- and medium-sized nucleic acids are best separated using polyacrylamide gel electrophoresis while larger molecules are separated on gels of agarose which have the largest pore size.

Agarose Gels

Preparation of Gel

1. Assemble the gel mold.
2. Add the weighed amount of agarose to the volume of buffer (1X TAE) needed to fill the mold.
3. Heat in a microwave oven until the agarose has dissolved.
4. Cool the agarose solution to about 50 °C, add ethidium bromide and swirl to mix, then pour into the mold and immediately place the comb in position.
5. When the gel has completely cooled and set (30 min), remove the comb and place the gel in the electrophoresis apparatus.
6. Add sufficient buffer to fill the electrode chamber and cover the gel to a depth of about 1 mm.
7. Load the samples and electrophorese at appropriate voltage.

10X Loading Buffer

- 50% (v/v) glycerol
- 0.5% (w/v) bromophenol blue
0.5% (w/v) xylene cyanol

**50X TAE Buffer**

- 242 g Tris base
- 57.1 ml Glacial acetic acid
- 100 ml 0.5 M EDTA pH 8.0

Dilute to 1 l

**Ethidium Bromide**

- 10 mg/ml

To prestain gel, add 1 µl /20 ml gel mixture.

To prestain gel buffer, add 10 µl to 3 l.

**Recovery of DNA from Agarose Gels onto DEAE Membrane**

**Introduction**

An incision is made in the gel just ahead of the band of interest and a strip of DEAE paper put in place. Electrophoresis is resumed until all of ethidium-staining material has transferred from the gel to the paper. The DNA is eluted with a high salt buffer.

**Preparation of DEAE Membrane**

1. Cut the DEAE membrane into strips as long as your band and 6 mm wide.
2. Soak in 10 mM EDTA pH 7.6 for 10 min.
3. Pour off EDTA solution and replace it with 0.5 M NaOH for 5 min.
4. Wash 5 times with double-distilled water. Membrane can be
prepared in advance and stored in water at 4 °C for later use.

Transfer Procedure

1. Prepare and run the agarose gel in a UV-transparent mold.

2. Identify the band(s) of interest and make an incision in the agarose on the anode side of the band using a scalpel blade. Open the slit in the gel and slide in a piece of prewetted DEAE paper.

3. Resume electrophoresis and monitor DNA migration by observing with the transluminator every few minutes. This is critical if several bands are closely spaced and unique fragments are to be isolated. The fluorescent band will disappear from the gel and be adsorbed onto the paper.

4. Place the DEAE paper to which DNA is bound in a 1.5 ml microcentrifuge tube and add 500 ul of high salt buffer to cover the area of paper where DNA is bound.

5. Incubate for 20 min in a 65 °C water bath.

6. Transfer the supernatant containing the DNA to a fresh 1.5 ml tube.

7. Add 500 ul of high salt buffer to the tube with DEAE paper and incubate for another 20 min at 65 °C.

8. Transfer the supernatant to a fresh 1.5 tube and extract with 250 ul of water-saturated phenol and 125 ul of chloroform-isoamyl alcohol (24:1).

9. Centrifuge for 4 min and transfer the aqueous layer to a fresh 1.5
ml tube.

10. Add 0.1 vol of 8 M ammonium acetate and 2 volumes of absolute ethanol and incubate at -80 °C for 1 h.

11. Remove the tubes from the deep freezer, let sit at RT for a few minutes, and centrifuge for 10-15 min.

12. Discard the supernatant and rinse the pellet with 200 ul of 70% ethanol (4 min).

13. Decant ethanol, blot on paper towel and dry in speedvac for approx. 10 min.

14. Resuspend the DNA insert in 20 ul of TE buffer and combine the fractions containing the same insert.

15. Electrophorese 5 ul DNA solution on a mini gel to assess the amount of DNA recovered.

**Solution**

**High Salt Buffer, pH 7.4:**

20 mM Tris pH 8.0

1 M NaCl

0.1 mM EDTA

This is the final concentration of the buffer.
APPENDIX 11
ISOLATION AND PURIFICATION OF RNA

Introduction

The most important consideration in the preparation of RNA is to rapidly and efficiently inhibit the endogenous ribonucleases which are present in virtually all living cells. Another important concern in the preparation of RNA is to avoid accidental introduction of trace amounts of ribonucleases from hands, glassware and solutions. It is therefore imperative to:

1. use gloves throughout all procedures.
2. autoclave reagents that need to be sterile.
3. use only sterile tubes, pipettes, tips and glassware.

Isolation of Total Cellular RNA from Luteal Tissue

Procedure

1. Label 50 ml conical centrifuge tubes and let sit on ice.
2. Add 10 ml of cold guanidinium thiocyanate to each tube.
3. Add 1 ml of 2 M sodium acetate (pH 5.0) to each tube and mix gently.
4. Add 1 g of fresh or frozen tissue (cut into pieces) to each tube.
5. Add 78 ul of 2-mercaptoethanol to each tube.
6. Homogenize tissue with a polytron tissue homogenizer for three to four 5 sec bursts. Keep tissue on ice during homogenization. Rinse the polytron head between samples.


8. Centrifuge at 4000 rpm for 15 min.

9. Transfer upper phase to a fresh 50 ml tube. Be careful not to take any of the bottom layer.

10. Add 10 ml of isopropanol to precipitate RNA. Mix gently and place in a -80°C freezer for 1 h.

11. Centrifuge at 4000 rpm for 10 min and discard the supernatant.

12. Resuspend the pellet in 2 ml of 4 M LiCl. Dislodge RNA pellet from bottom of the tube using a sterile transfer pipette. RNA does not go into solution.

13. Centrifuge at 4000 rpm for 10-15 min to repellet.

14. Pour off the supernatant and blot on a clear paper towel.

15. Resuspend the pellet in 2 ml of RNA buffer (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5) and let sit at RT until RNA goes into solution. It takes about 15-40 min to go into solution.

16. Transfer content of the tube into a 15 ml conical tube.

17. Add 2 ml of chloroform and vortex for 15 sec.

18. Centrifuge at 4000 rpm for 10 min.
19. Collect upper phase with sterile pipet and transfer into a fresh 15 ml conical tube.

20. Add 200 ul of 2 M sodium acetate (pH 5.0) and mix gently.

21. Add 2 ml of isopropanol and mix.

22. Store overnight in a - 80 °C freezer.

23. Centrifuge at 4000 rpm for 30 min. Insoluble pellet should contain pure RNA after centrifugation.

24. Pour off supernatant, blot and add approximately 1 ml of cold 70% ethanol to wash pellet. Recentrifuge at 4000 rpm for 10 min. Discard supernatant and blot on adsorbent paper. Air dry 12-15 min.

25. Resuspend the pellet in 100-200 ul sterile H2O. Let sit at RT for 10 min or until it dissolves. Mix gently and transfer to 1.5 ml bullet tubes.

26. Pipet 5 ul of sample to a 12 X 75 test tube.


** To calculate ug RNA use the following equation:

\[ \text{O.D. 260 (___)} \times \text{Dilution (___)} \times 33 \, \text{ug/ml} \times \text{Total sample volume} \]

\[ = \text{___ Total ug RNA.} \]

At 260 nm, RNA has a refractive index of 33 (ie 33 μg for each OD
DNA has a refractive index of 40.

**

To determine RNA purity: calculate 260:280 ratio.

a. Ratios between 1.8 - 2.0 are good.

b. Ratios less than 1.2 may indicate substantial protein contamination.

** Solutions for RNA Extraction

** 4 M Guanidinium thiocynate:

4 M guanidinium thiocyanate (236.4 g)

25 mM sodium citrate (3.7 g)

0.5 % (w/v) sarkosyl (2.5 g)

pH to 7.0

Distilled water to 500 ml

Autoclave and store in dark bottle at 4 °C

** 4 M LiCl:

Add 42.4 g LiCl

Distilled H₂O to 250 ml

Autoclave and store at 4 °C

** RNA buffer, pH 7.5:

10 mM Tris-HCl, pH 7.5

1 mM EDTA

0.5% (w/v) SDS

Autoclave and store at -20 °C
2 M Sodium acetate, pH 5.0:

16.4 g Sodium acetate

Add 80 ml of acetic acid

pH to 4.0 (difficult to get pH to 4)

Add water to 100 ml

Autoclave and store at -20 °C

Water Saturated Phenol

Melt phenol at 65 °C.

Add an equal volume of distilled water.

Store at 4 °C. The phenol settles at the bottom.
APPENDIX 12
NUCLEIC ACID LABELLING

Nick Translation

Introduction

E. coli DNA polymerase I adds nucleotide residues to the 3' hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked. In addition, by virtue of its 5'-> 3' exonucleolytic activity, the enzyme can remove nucleotides from the 5' side of the nick. The simultaneous elimination of nucleotides from the 5' side and the addition of nucleotides to the 3' side results in movement of the nick along the DNA.

Procedure

1. Add the following components to a 500 ul Eppendorf tube:

   DNA to be labeled 500 ng
   Nucleotide mix buffer (kit) 20 ul
   [α-32P]dCTP 5 ul
   Enzyme (kit) 10 ul
   H2O (to a total volume of 100 ul)
2. Mix lightly and incubate at 14 C for 1 h.

3. Add an equal volume of phenol (100 ul) and vortex.

4. Run the entire fraction over a Sephadex G-50 column which has been equilibrated in TE buffer.

5. Check radioactivity of fractions with a Geiger counter.

6. Save fractions containing the first peak of radioactivity for hybridization.
APPENDIX 13
NORTHERN BLOTTING AND HYBRIDIZATION

Introduction

RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to nitrocellulose or nylon membrane. The RNA of interest is then located by hybridization with radiolabeled DNA or RNA followed by autoradiography.

A. RNA Denaturation

1. Aliquot total RNA (10-30 ug) into 0.5 ml tubes.
2. Lyophilize samples in speed vac for 15 to 20 min.
3. Add 15 ul sample denaturation buffer to each tube.
4. Let samples sit on ice for at least one hour. While denaturing the samples, set up 1.5 % agarose gel as described in step B.

B. Setting up a 1.5 % Agarose Gel

1. Weigh out 3.0 g of high melt agarose and place in a 500 ml glass beaker.
2. In a graduated cylinder, mix 8 ml running buffer (25X), 158 ml distilled water and add this to the beaker with agarose.
3. Heat the solution in a microwave for 1 to 2 min. Repeat this step
until agarose is completely dissolved.

4. Add 32.1 ml 37% formaldehyde to the agarose solution and mix.

5. When agarose is sufficiently cool, pour into the gel holder. Make sure there are no air bubbles within the gel or near the comb.

6. Allow the gel to polymerize for at least 1 h in the hood.

C. Loading and Running RNA samples

1. Pour the running buffer (1X) in electrophoresis unit and place the gel in the caster.

2. Carefully remove the comb and add enough buffer to cover the gel.

3. Turn on the pump and get all air bubbles out of the line and then turn off.

4. Heat denature RNA samples at 65 °C for 15 min.

5. Cool quickly on ice and add 5 ul of loading buffer.

6. With the pump turned off, add samples to the wells. The circulation unit should be connected so that the buffer runs from the anode (black) to the cathode (red).

7. Electrophorese samples at 100 V until the dye front is in the gel (20 min).

8. Turn on the pump and reduce the voltage to 24 V to run overnight.

9. The next morning, with the pump still running, add 10-15 ul of ethidium bromide (10 mg/ml) and let stain for 30 to 60 min.

10. Turn the pump off and check RNA quality on UV light box. The 28s
and 18s ribosomal RNA bands should be intact.

D. **Northern Blotting**

RNA transfer was done using the TurboBlotter and Blotting Assembly.

1. RNA gels run rinsed in 2.2 M formaldehyde should be rinsed four times in deionized water.
2. Measure the gel and cut a piece of Nylon filter paper to the exact size, using a pair of sharp scissors. Soak transfer membrane in distilled water for 15 min.
3. Pour off water and soak the membrane in 20X SSC. Cut another piece of Whatman paper to the same size as the nylon membrane.
4. Place "stack tray" of transfer device on bench, making sure it is level.
5. Place 20 sheets of dry GB004 blotting paper (thick) in stack tray.
6. Place 4 sheets of dry GB002 blotting paper (thin) on top stack.
7. Place one sheet of GB002 blotting paper, prewet in transfer buffer on stack.
8. Place transfer membrane on stack.
9. Cover the membrane with agarose gel, cut the gel to the size of the membrane, making sure there are no air bubbles between the gel and the membrane.
10. Wet the top of the gel with transfer buffer and place 3 sheets of GB002 blotting paper, presoaked in transfer buffer on top of the
gel.

11. Attach the "buffer tray" of the transfer device to the bottom tray using the circular allignment buttons to align both trays.

12. Fill the buffer tray with transfer buffer.

13. Start transfer by connecting the gel stack with the buffer tray using the pre-cut "buffer wick" (included in each blotter stack), presoaked in transfer buffer. Place the wick across the stack so that the short dimension of the wick completely covers the blotting stack and both ends of the long dimension extend into the buffer tray. Place the "wick cover" on top of the stack to prevent evaporation. Make sure the edges of the wick are immersed in the transfer buffer.

14. Continue the transfer for at least 5 h or overnight.

15. Dismantle the blot and check the efficiency of RNA transfer on a UV light box. Mark the position of the wells and ribosomal RNA bands on the nylon membrane with a blunt pencil.

10. To immobilize the RNA, place the nylon membrane between two sheets of Whatman paper and bake at 80 °C for 2 h in a vacuum oven, or expose blot to ultraviolet light on a light box for 90 sec. The membrane can be prehybridized immediately or stored dry in a cool place.
E. **Hybridization and Autoradiography**

1. Prehybridize the nylon membrane for 1 to 2 h at 42 °C in prehybridization buffer (see below).

2. Discard the prehybridization solution and add the hybridization buffer (see below).

3. Add the denatured radiolabelled probe (boiled for 5 min) directly to the hybridization solution and continue the incubation for 24 h.

4. Wash the membrane twice (30 and 15 min) at 42 °C in 2 X SSC, 0.1% SDS, followed by two washes of 15 min each at 42 °C in 0.1 X SSC, 0.1% SDS.

5. Establish an autoradiograph by exposing the membrane for 24-48 h to X-ray film (Dupont, ) at -70 °C with an intensifying screen.

**Solutions**

**25X Running Buffer, pH 7.0**

Make 0.5 M stock solutions of mono and dibasic phosphate:

A: 60 g NaH$_2$PO$_4$ (mono, FW=120.0)

B: 71 g Na$_2$HPO$_4$ (di, FW=141.96)

Mix 39 ml of solution A and 61 ml of solution B H$_2$O to 200 ml. Autoclave and store at room temperature.
10X Denaturing Buffer, pH 7.0

6.76 g Hepes (FW=238.3)
0.68 g Sodium citrate (FW=136.1)
0.37 g EDTA
H₂O to 100 ml

Autoclave and store at -20 °C wrapped in foil

Sample Denaturing Buffer, pH 7.5

0.72 ml 10X denaturing buffer
3.40 ml 90% formamide
1.07 ml 37% formaldehyde
0.81 ml H₂O

Filter-sterilize and store at -20 °C

Loading Buffer

0.4 ml 25X running buffer (20 mM)
5.0 ml glycerol (50%)
4.6 ml H₂O
5.0 mg bromophenol blue (0.05% w/v)

20X SSC, pH 7.0

3 M Sodium chloride (175 g/l)
0.3 M Sodium citrate (88 g/l)

pH to 7.0 with 1 M HCl
**50X Denhardt Solution, 500 ml**

5 g Ficoll  
5 g Polynynlypyrrolidone  
5 g BSA  

Filter-sterilize and store at -20 °C

**Prehybridization Buffer, 50 ml**

5.0 ml 50X Denhardt solution  
10.0 ml 20X SSC  
4.0 ml 0.5 M phosphate, pH 6.5  
0.5 ml 10% SDS  
1.0 ml Yeast RNA (12.5 mg/ml)  
25.0 ml Formamide  
4.5 ml H₂O  

Filter-sterilize and store at -20 °C

**Hybridization Buffer, 50 ml**

1.0 ml 50X Denhardt solution  
10.0 ml 20X SSC  
4.0 ml 0.5 M phosphate, pH 6.5  
0.5 ml 10% SDS  
1.0 ml Yeast RNA (12.5 mg/ml)  
25.0 ml Formamide  
8.5 ml H₂O
Filter-sterilize and store at -20 °C

**Wash Solutions**

Low stringency 2X SSC, 0.1% SDS

High stringency 0.1X SSC, 0.1% SDS

All reagents were of electrophoresis grade (Fisher Biomedicals).
APPENDIX 14
RNA DOT BLOT HYBRIDIZATION

A. Sample Preparation
1. Pipet 2.5, 5, 10 or 20 μg of each RNA sample into sterile bullet tubes. Speed vac the samples.
2. Dilute the RNA with 250 μl of Denaturation Buffer.
3. Incubate the samples in 65 °C water bath for 5 min.
4. Add 250 μl of 20X SSC to each tube. Vortex mix briefly for 5 to 10 sec. The sample is ready to load.

B. Filter Preparation
1. Cut a piece of BioTrans nylon membrane to the appropriate size and soak the membrane in water and then in 10X SSC.
2. Soak the Schleicher & Schiell baking paper for the dot apparatus in water and then in 10X SSC.
3. Place one layer of baking paper on the blotting unit. Place the Bio Trans membrane on top of the baking paper.
4. Clamp the blotting unit together.

C. Loading RNA Samples
1. Prefilter the wells with 500 μl of 10X SSC. Turn on the vacuum so
the buffer moves through the wells slowly.

2. Load the RNA samples and elute slowly. Turn on the vacuum only after all RNA samples have been loaded.

3. Once all samples have filtered through, wash each well with 500 µl of 20X SSC.

4. Disassemble the apparatus, mark filter for orientation and allow it to air dry.

5. Place the membrane between two sheets of Whatman paper and bake it at 80°C for 2 h or expose the membrane to ultraviolet light for 90 sec.

D. Hybridization

1. Prehybridize each filter in prehybridization buffer at 42°C for 2 h.

2. Hybridize each filter with hybridization buffer and the appropriate probe. Incubate at 42°C overnight.

3. Visualize RNA dots by autoradiography at -70°C.

E. Solutions

Denaturation Buffer, pH 7.0:

20 mM Tris

50 % Formamide

6 % Formaldehyde

This is the final concentration. Filter-sterilize.
Yeast RNA

1. Weigh 400 yeast RNA (Ribonucleinsaure, Boehringer Mannheim) and place in a 50 ml conical tube. Prepare 4 tube simultaneously.

2. Add 20 ml of TE (pH 7.5) to obtain a 20 mg/ml solution.

3. Add 0.026 volumes (520 µl) 8 M ammonium acetate. Mix and place at 65°C in a water bath for 2-3 min until RNA is in solution.

4. Add 0.75 volumes phenol (15 ml) and 0.25 volumes chloroform-isoamyl alcohol (5 ml), mix and spin at 4000 rpm for 15 min (Sorvall RC5B with HS-4 rotor).

5. Remove upper phase with a sterile transfer pipet and place in a fresh 50-ml conical tube. 60% of the volume (24 ml) is usually recovered as the upper phase.

6. Add 0.75 volumes of phenol (18 ml) and 0.25 volumes of chloroform-isoamyl alcohol (6 ml), mix and centrifuge at 4000 rpm for 15 min.

7. Remove upper phase (usually about 24 ml) and divide into halves and place in a new set of 50-ml conical tubes. Add 0.1 volume (1.2 ml) 8 M ammonium acetate and mix. Freeze at -80 C for 1 h.

8. Centrifuge at 4000 rpm for 30 min.

9. Discard supernatant, invert tube and allow to dry for 5 min.

10. Wash with 2 ml 70% ethanol. Centrifuge at 4000 rpm for 10 min.
11. Resuspend in original volume with TE (10 ml/tube). Once in solution, combine all fractions, mix and aliquot in 15 ml conical tubes.

12. Store at -20 C.
APPENDIX 15
IMMUNOHISTOCHEMICAL LOCALIZATION OF APO E

Procedure

Immunostaining was done using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA).

1. Deparaffinize and hydrate tissue sections through xylenes or other clearing agents and graded alcohol series.

2. Rinse for 5 min in distilled water.

3. If quenching of endogenous peroxidase activity is required, incubate sections for 30 min in 0.3% H₂O₂ in methanol. Skip this step if endogenous peroxidase is not a problem.

4. Wash in buffer for 20 min.

5. Incubate sections for 20 min with diluted normal serum from the species in which the secondary antibody is made. In cases where non-specific staining is not a problem, skip steps 5 and 6.

6. Blot excess serum from sections.

7. Incubate sections overnight with primary Apo E antiserum diluted 1:500 in buffer.

8. Wash slides for 10 min in buffer.
9. Incubate sections for 30 min with diluted biotinylated antibody solution.

10. Wash slices for 10 min in buffer.

11. Incubate sections for 30-60 min with VECTASTAIN ABC reagent.

12. Wash slides for 10 min in buffer.

13. Incubate sections for 2-7 min in peroxidase substrate solution.

14. Wash sections for 5 min in tap water.

15. Counterstain, clear and mount.

**Solutions**

**Bouins Fixative for Light Microscopy:**

- 750 ml saturated picric acid
- 250 ml glacial acetic acid

**Fixative for Electron Microscopy (0.1 M sodium cacodylate, 1% glutaraldehyde):**

Prepare 0.2 M sodium cacodylate (0.856 g/ 20 ml distilled water).

Adjust pH to 7.4

Add 5 ml 8% glutaraldehyde

Add 15 ml distilled water

Make up to 40 ml with distilled water. Store at 4°C
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Legend Key for Raw Data:

S = Status; 1 = Cycle, 2 = Pregnancy
D = Day of estrous cycle or pregnancy
CLWt = Mass of whole corpus luteum (g)
Inc = Percent incorporation of radiolabel
TCA = TCA-precipitable radioactivity (dpm)
Prot = Total protein concentration (µg/ml) of retentate
P4 = Plasma progesterone concentration (ng/ml)
P6 = Density of protein 6 on fluorograph
P7 = Density of protein 7 on fluorograph
TIMP1 = Density of TIMP-1 on fluorograph
ApoA1 = Density of Apo A1 on fluorograph
TIMP2/SOD = Density of TIMP-2/SOD complex on fluorograph
APPENDIX 17
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Legend Key for Raw Data:

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5 = days 90-120, 6 = days 170-180, 7 = days > 215
ApoA1 = Intensity of dot blot for Apo A-1 mRNA
TIMP1 = Intensity of dot blot for TIMP-1 mRNA
TIMP2 = Intensity of dot blot for TIMP-2 mRNA
ActA1 = Intensity of dot blot for Apo A-1 reprobed with β-actin
RA1 = Intensity of Apo A-1 mRNA relative to β-actin
ActT1 = Intensity of dot blot for TIMP-1 reprobed with β-actin
RT1 = Intensity of TIMP-1 mRNA relative to β-actin
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S = Status; 1 = Cycle, 2 = Pregnancy
SOD = Intensity of dot blot for Mn SOD mRNA
ActSOD = Intensity of dot blot for Mn SOD reprobed with β-actin
RSOD = Intensity of Mn SOD mRNA relative to β-actin
# APPENDIX 18

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Legend key for Data of Protein Secretion \500 mg Tissue

S = status
St = stage
D = day
dA1 = Apo A-1 secretion \500 mg tissue
dT1 = TIMP-1 secretion \500 mg tissue
dT2S = TIMP-2\SOD secretion \500 mg tissue
dp58 = Protein 6 secretion \500 mg tissue
dp44 = Protein 7 secretion \500 mg tissue
BIOGRAPHICAL SKETCH

Florence Maboh Ndikum-Moffor is a Cameroonian and was born May 26, ______, to Samuel Ndango Mutia and Rufina Manyi Mutia in Enugu, Nigeria. She received her Bachelor of Science degree in chemistry from the University of Ibadan, Nigeria in November 1980, and a Master of Science degree in chemical pathology from the same university. The author then worked as a research officer with the Institute of Animal and Veterinary Research, Cameroon, from 1983 to 1991, when she enrolled in the doctoral program in the Animal Science Department at the University of Florida under the supervision of Dr. Michael J. Fields (Professor). After completion of her degree she would like to continue her training in the biological sciences as a postdoctoral fellow. Florence is married to Gaston Ndikoum Moffor and they are blessed with three children, Kongwenebime 11, Koga 8, and Mandi 3.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael J. Fields, Chair
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lynn H. Larkin
Professor of Anatomy and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rosalia C.M. Simmen
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William C. Buhi
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1995

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