BOVINE TROPHOBLAST PROTEIN-1:
CHEMICAL CHARACTERISTICS AND ANTILUTEOLYTIC
EFFECTS ON UTERINE AND OVARIAN FUNCTION

By

STEPHEN DEAN HELMER

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988
ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to the chairman of his advisory committee, Dr. William W. Thatcher, for his continuous guidance, support and most importantly, his friendship throughout his doctoral program. Special thanks to Dr. Peter J. Hansen for insight and friendship. He also expresses thanks to the remaining members of his advisory committee Drs. Fuller W. Bazer and William C. Buhi for their assistance during various phases of the program of study.

Further appreciation goes to Dr. R. Micheal Roberts for invaluable guidance during the author's first two years of study, and to Dr. Russel V. Anthony for advice, assistance and constant friendship.

Special thanks go to Austin Greene, Dale Hissem and Tom Bruce for invaluable assistance in managing and making available cattle for the study. For their willingness to extend to the author their clinical expertise, thanks are extended to Drs. Martin Drost and Scott Norman.

The author also thanks Dr. Timothy Gross, Jesse Johnson, Mary Ellen Hissem and Leslie Smith for their expert technical advice and assistance. Thanks also go to Mr. Larry Eubanks for use of the slaughter facilities.

For their continual support and friendship the author extends special thanks to the graduate students and postdoctoral fellows who
are too numerous to acknowledge individually, but made the author's graduate program memorable. In particular, thanks go to John McDermott, Jeffery Knickerbocker, D. James Putney, Lokenga Badinga, Deanne Morse, Matt Lucy, Jerry Malayer and Claire Plante, whom the author feels privileged to have known.

The author expresses sincere appreciation to his parents, Dean P. Helmer and Marilyn T. Helmer, and to Mr. and Mrs. Frederick C. Pareis for their continued love and support.

Special thanks and appreciation are expressed to the author's wife, and best friend, Judith P. Helmer, for her unending love and support.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ....................................................................................... ii

LIST OF TABLES .............................................................................................. vi

LIST OF FIGURES .............................................................................................. vii

ABSTRACT ........................................................................................................... ix

CHAPTERS

1 LITERATURE REVIEW .................................................................................. 1
   Introduction ................................................................................................... 1
   The Bovine Estrous Cycle ........................................................................... 2
   Physiology and Endocrinology of the Ovary ............................................. 3
   Uterine Regulation of CL Lifespan During the Estrous Cycle ............... 29
   Uterine Regulation of CL Lifespan During Pregnancy ............................ 39
   Developement of the Bovine Conceptus ..................................................... 43
   Timing of the Conceptus Signal Relative to "Maternal Recognition of Pregnancy" .............................................................. 44

2 IDENTIFICATION OF BOVINE TROPHOBLAST PROTEIN-1, A SECRETORY PROTEIN IMMUNOLOGICALLY RELATED TO OVINE TROPHOBLAST PROTEIN-1 .............................................................. 66
   Introduction ................................................................................................... 66
   Materials and Methods ............................................................................. 68
   Results .......................................................................................................... 74
   Discussion ...................................................................................................... 82

3 DIFFERENTIAL GLYCOSYLATION OF THE COMPONENTS OF THE BOVINE TROPHOBLAST PROTEIN-1 COMPLEX .................................................................................. 87
   Introduction ................................................................................................... 87
   Materials and Methods ............................................................................. 88
   Results .......................................................................................................... 92
   Discussion ...................................................................................................... 98
4 INTRAUTERINE INFUSION OF PURIFIED BOVINE TROPHOBLAST PROTEIN-1 COMPLEX EXERTS AN ANTILUTEOLYTIC EFFECT AND EXTENDS CORPUS LUTEUM LIFESPAN IN CYCLIC CATTLE

Introduction.................................................................................103
Materials and Methods.................................................................104
Results..........................................................................................116
Discussion.....................................................................................139

5 BOVINE TROPHOBLAST PROTEIN-1 COMPLEX ALTERS ENDOMETRIAL PROTEIN AND PROSTAGLANDIN SECRETION AND INDUCES AN INTRACELLULAR INHIBITOR OF PROSTAGLANDIN SYNTHESIS IN VITRO

Introduction..................................................................................142
Materials and Methods.................................................................144
Results..........................................................................................152
Discussion.....................................................................................158

6 GENERAL CONCLUSIONS..........................................................163

REFERENCES..................................................................................175

BIOGAPHICAL SKETCH.................................................................200
LIST OF TABLES

Table                                      Page

4-1 Percent incorporation of $[^3\text{H}]$-leucine into nondialyzable protein and protein content for the first, second, third and fourth 24 h culture periods of conceptuses........117

4-2 Least square means $\pm$ sem for characteristics of estrous cycles for cows treated with BSA, bCSP or bTP-1 complex........131

4-3 Least square means $\pm$ sem of plasma PGF concentrations and residual variances for BSA, bCSP and bTP-1 treated cattle..............................136

5-1 Analysis of variance for incorporation of $[^3\text{H}]$-leucine into secretory proteins by endometrial explants treated with BSA, bCSP or bTP-1 complex.................................150

5-2 Analysis of variance for PGF and PGE in medium for endometrial explants incubated with BSA, bCSP or bTP-1........151

5-3 Least square means$^a$ of concentrations of PGF and PGE secreted into medium of day 17 endometrial explants incubated for 24 h with medium containing 4.8 $\mu$g BSA/ml, 12.7 $\mu$g bCSP/ml or 1 $\mu$g bTP-1/ml................................153

5-4 Least square means$^a$ for incorporation of $[^3\text{H}]$-leucine into TCA-precipitable macromolecules in medium (dpm/250 mg/15 ml) and tissue (dpm/250 mg/24 h) from endometrial explants incubated for 24 h with medium containing no BSA, 4.8 $\mu$g BSA/ml, 12.7 $\mu$g bCSP/ml or 1 $\mu$g bTP-1/ml........157
### LIST OF FIGURES

**Figure** | **Page**
---|---
2-1  | Ouchterlony double-immunodiffusion analysis of conceptus secretory proteins from (a) sheep and (b) cattle. 75
2-2  | Ouchterlony double-immunodiffusion analysis of bovine and ovine conceptus secretory proteins. 76
2-3  | Solid phase radiobinding assay of conceptus secretory proteins. 77
2-4  | Analysis of immunoprecipitates from conceptus secretory proteins of cows and sheep by one-dimensional polyacrylamide gel electrophoresis and fluorography. 79
2-5  | Analysis of bovine CSP by two-dimensional polyacrylamide gel electrophoresis and fluorography. 81
2-6  | Electrophoretic analysis of cell-free translation products of RNA isolated from cattle conceptuses. 83
3-1  | Fluorograph of SDS-PAGE of conceptus supernatants from a conceptus cultured in medium containing 100 μCi [3H] glucosamine. 93
3-2  | Fluorograph of SDS-PAGE of conceptus supernatants from conceptuses cultured in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of tunicamycin for 24 h. 95
3-3  | Fluorograph of SDS-PAGE of conceptus supernatants from conceptuses cultured in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of DMM for 24 h. 96
3-4  | Fluorograph of SDS-PAGE of conceptus proteins treated with Endo H. 97
3-5  | Concanavalin A-Sepharose 4B lectin chromatography of bTP-1. 99
4-1  | HPLC gel filtration profiles of radiolabelled conceptus-conditioned medium from the first, second, third and fourth days of culture of a conceptus. 119
4-2 Fluorograph of electrophoretogram of bCSPs subjected to 40% or 50% saturated ammonium sulfate (SAS) precipitation...122

4-3 Fluorograph of electrophoretogram of bCSP after separation by HPLC gel filtration..........................124

4-4 Silver-stained, two-dimensional electrophoretogram of highly purified bTP-1 complex (16 µg, top left panel); a gel which had been run with sample buffer, but no protein (top right panel); total array of bCSPs (100 µg, bottom left panel and bovine serum albumin (20 µg, bottom right panel)........................................127

4-5 Autoradiograph of 2-D SDS-PAGE of purified bTP-1 complex (8 µg/ml) transferred to nitrocellulose and immunoblotted with a) rabbit anti-oTP-1 antiserum (1:100) or b) normal rabbit serum........................................130

4-6 Progesterone profiles of cattle receiving intrauterine infusion of bovine serum albumin (BSA, top panel); bCSP (middle panel) or purified bTP-1 complex (bottom panel) from days 15.5 to 21 of an estrous cycle.....................134

4-7 Representative profiles for plasma PGF of a BSA, bCSP, and bTP-1 treated cow sampled every 15 min on day 19 after estrus.........................................................138

5-1 Least squares means (pooled sem=0.20) for PG synthesis by the prostaglandin generating system in the presence of cytosolic supernatants from day 17 endometrial explants which had been treated with no BSA, 4.8 µg BSA/ml, 12 µg bCSP/ml or 1 µg bTP-1/ml for 24 h.......................................................155

6-1 Proposed model for differential glycosylation of the components of the bTP-1 complex........................168

6-2 Proposed model for the bovine antiluteolytic pathway during early pregnancy..........................173
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

BOVINE TROPHOBLAST PROTEIN-1: CHEMICAL CHARACTERISTICS AND ANTILUTEOLYTIC EFFECTS ON UTERINE AND OVARIAN FUNCTION

by

STEPHEN DEAN HELMER

AUGUST 1988

Chairman: William W. Thatcher
Major Department: Animal Science

The conceptus must "signal" its presence to extend functional lifespan of the corpus luteum (CL), a requirement of pregnancy maintenance. Conceptus signals in both the ewe and cow are proteinaceous in nature. In sheep this signal is ovine trophoblast protein-1 (oTP-1). The antiluteolytic mechanisms for the ewe and cow are similar because reciprocal transfer of extraembryonic membranes into the uterus of sheep and cattle extend CL function in some cases. However, the putative bovine antiluteolytic signal has not been isolated. The research described in this dissertation was carried out in an attempt to extend our understanding of conceptus mediated antiluteolytic mechanisms in the cow.

Components of bovine and ovine conceptus secretory proteins (bCSP, oCSP) cross-react with antiserum directed against oTP-1. The immunologically cross-reactive components of bCSP (7 variants) are defined as the bovine trophoblast protein-1 complex (bTP-1). This
complex differs from oTP-1 in size (22 to 26 kDa vs. 19 kDa) and isoelectric points (pI 6.5-6.7 vs. 5.3-5.7). The size differences result from the fact that bTP-1, unlike oTP-1, is glycosylated. The 22 and 26 kDa species of bTP-1 were high-mannose and complex-type glycoproteins, respectively. The deglycosylated form of bTP-1 migrated as an 18 kDa species during electrophoresis, a value close to molecular weight estimates of oTP-1.

Antiluteolytic effects of bTP-1 were examined. Interestrous intervals were longer for cyclic cows receiving intrauterine infusions of bTP-1 compared to bCSP or BSA. A tendency toward attenuated uterine PGF secretion was noted with a significant decrease in residual variance in samples of bTP-1 treated cows because luteolytic-type pulses of PGF were diminished.

In a separate experiment, bTP-1 and bCSP attenuated PGF release by endometrial explant cultures and induced an intracellular inhibitor of PG-synthesizing enzymes. Protein secretion also was decreased by bTP-1 and bCSP treated explants.

Collectively, these data indicate that bTP-1 is the antiluteolytic component of bCSP. This conceptus signal regulates PGF secretion by inducing an inhibitor of PG synthesizing enzymes in cattle to allow for extension of CL function and associated progesterone secretion.
CHAPTER 1
LITERATURE REVIEW

Introduction

The cow is afforded an opportunity to become pregnant every 21 days. This opportunity results from the release of an ovum from the ovary at a time favorable for fertilization and subsequent development in utero. The sequence of events leading up to and including ovulation are regulated by complex mechanisms and have been studied extensively in the past. Recently, there has been a greater understanding of the mechanisms of intercommunication between the conceptus and maternal unit. That the conceptus must "signal" the maternal unit or uterine environment is evident because CL regression and recurrent estrous cycles continue in the absence of pregnancy. The nature of this conceptus derived, antiluteolytic "signal" appears to be proteinaceous in nature. However, its identity, biological characteristics, and mechanism of action are not fully understood. The research described in this dissertation was to develop a greater understanding of the nature and mechanism of action of these putative conceptus "signals" in initiating the sequence of events leading to CL maintenance and successful establishment of pregnancy.
The Bovine Estrous Cycle

Cattle display a periodicity of sexual behavior which has formed the basis for an exhaustive quest for knowledge. This period of sexual receptivity, known as estrus, is a recurrent event and delineates the boundaries of time referred to as the estrous cycle. Observations of the cyclic nature of domestic cattle were made as early as 1876 by Wallace (as cited by Marshall, 1922). The estrous cycle, or interestrous interval, averages 21 days (Hansel et al., 1973). Cattle display behavioral signs of estrus for an average period of 16.9 ± 4.9 hours (h) (Schams et al., 1977), but variability between and within breeds can be high (for reviews, see Wishart, 1972). Estrous cycle lengths may be more repeatable on an individual animal basis. Wishart (1972) reported that individual animal variation was less than 2 days in 77.3 percent of 211 estrous cycles. Chapman & Casida (1937) reported repeatability of estrous cycle lengths to be 0.41. In a different study, the repeatability estimate, described as the regularity of the occurrence of estrus, was 0.18 (Pou et al., 1953).

The estrous cycle can be conveniently divided into four phases: proestrus, estrus, metestrus and diestrus. The behavioral, endocrine and biochemical changes which occur during the estrous cycle are regulated by complex interactions of the hypothalamus, pituitary, ovary, and uterus. This complex process results in release of a fertilizable ovum, which may establish itself in utero and result in the birth of young. An understanding of these processes might be best obtained through a discussion of follicular development.
Physiology and Endocrinology of the Ovary

Dynamics of Follicular Growth

Two endocrine structures relevant to reproductive physiology are found on the ovary, 1) the follicle and 2) corpus luteum (CL). Follicles are present at all stages of the estrous cycle (Matton et al., 1981) and can be classified according to their degree of development. The following section on follicle development to the mature Graafian follicle is based upon histological observations of Rajakoski (1960), Lobel & Levy (1968) and Marion et al. (1968). They arise as primordial follicles which consist of the ovum and one layer of epithelial cells. The primordial follicles comprise the pool of all follicles present in the ovary at birth. These are depleted as individual follicles are recruited to grow (Marion & Gier, 1971). Once stimulated to grow, the cells surrounding the ovum, now referred to as granulosa cells, become cuboidal in shape and are known as primary follicles. Once mitosis of granulosa cells surrounding the ovum results in formation of several cell layers, it is designated a secondary follicle. During this time, the zona pellucida is formed and vascularization of the stroma surrounding the granulosa and basal lamina occurs. Further development to tertiary follicles is characterized by formation of a fluid-filled antrum within the mass of granulosa cells surrounding the ovum. As fluid accumulation proceeds, internal and external thecal cell layers become more organized and the ovum, surrounded by the corona radiata cells, becomes suspended by the cumulus oophorus granulosa cells within the
developing antrum. Mature antral follicles, generally greater than 10 mm in diameter, are designated as Graafian follicles.

It is believed that mature Graafian follicles arise as the result of waves of follicular development. Dynamics of the process of follicular recruitment, atresia and ovulation have been extensively reviewed (Rajakoski, 1960; Choudary et al., 1968; Dufour et al., 1972; Matton et al., 1981, Ireland & Roche, 1983a,b; Ireland, 1987). Early observations as to the timing of follicular waves yielded conflicting results. Rajakoski (1960) suggested that there were two "waves" of follicular growth during the bovine estrous cycle. The first of these was initiated on day 3 of the cycle and ended at mid-cycle with the development of an ovulatory sized follicle which eventually underwent atresia. A second wave of development begins around mid-cycle and ends in the formation of the preovulatory Graafian follicle. Contrary to these findings, Choudary et al. (1968) and Marion & Gier (1971) reported that follicular growth was continuous and independent of the phases of the cycle. They found that normal follicles greater than 5 mm in diameter were only present during the follicular phase. Large atretic follicles were present at all times in the follicular and luteal phases.

The biphasic theory of follicular development of Rajakoski (1960) has been supported by results of several other laboratories (Dufour et al., 1972; Matton et al., 1981; and Pierson & Ginther, 1984). Utilizing ultrasound to characterize follicular development, Pierson & Ginther (1984) reported that mean number of follicles among days differed for the 4-6 mm and greater than 10 mm categories. These
differences appeared to be due to: 1) an increase in 4-6 mm follicles at early diestrus which grow to ovulatory size and regressed at mid-diestrus and 2) an accelerated growth of the follicle destined to ovulate four days prior to ovulation. More recently, evidence for existence of three waves of follicular growth and selection have been reported (Fortune et al., 1988). Sirois & Fortune (1988), using ultrasonography, found that 7 of 10 heifers studied had three waves of follicular development with the third resulting in ovulation. The first two began on about days 1.9 and 9.4 with the ovulatory wave beginning on day 16.1.

Growth rates of follicles have also been studied (Lussier et al., 1987). They found that growth rate varied with follicular size. Follicle growth from 0.13-0.67 mm, 0.68-3.67 mm and 3.68-6.50 mm required 27, 6.8, and 7.8 d, respectively. The entire process (that period of development from recruitment to attainment of ovulatory size) requires a period of time equivalent to two estrous cycles. The relatively slow growth of small follicles suggests a selective control over their growth. This leads to a discussion of follicle dominance as related to the hormonal control of follicular development. The rapid rate of development for preovulatory sized follicles has lead to a further understanding of follicular dynamics.

Rapid replacement of large follicles has been noted by several laboratories (Dufour et al., 1972; Matton et al., 1981; Lussier et al., 1987). Dufour et al. (1972) and Matton et al. (1981) marked the largest and second largest follicles with India ink and determined if these remained the largest and second largest later in the cycle.
The results of these studies indicated that the ovulatory follicle could not be predicted prior to 4 days before ovulation.

Ovulation then results in a separation of ovum and associated cumulus oophorus and corona radiata cells from the follicle. The fate of the ovum and subsequent conceptus development will be discussed later. The thecal and granulosa cells of the follicle become the corpus luteum (CL) through cellular reorganization and proliferation under hormonal control of the pituitary.

**Dynamics of Corpus Luteum Development**

The corpus luteum develops from the cells of the follicle following ovulation. Donaldson & Hansel (1965a) reported that luteal cells are derived from both the theca interna and granulosa cells of the ovulatory follicle. Similar histological studies by Priedkalns et al. (1968) support this hypothesis. Luteinization is directed by the effects of luteinizing hormone (LH) from the anterior pituitary gland (Hansel, 1966).

Luteinization begins approximately 6 h after onset of estrus (Donaldson & Hansel, 1965a). Mitotic activity increases in both the thecal and granulosa layers, but is more frequent in the granulosa cells. Nuclei of granulosa cells enlarged during the first 4 days of the estrous cycle (estrus= day 0). The greater mitotic activity of the granulosa cells was associated with an increased ability to bind LH during this period (Niswender et al., 1981).

The remainder of this section is based upon the histological observations by Donaldson & Hansel (1965a) and Priedkalns et al. (1968) on development and regression of the CL.
Immediately following ovulation, which occurs 24 to 30 h after the preovulatory LH surge (day 1; Chenault et al., 1975; Schams et al., 1977; Hansel & Convey, 1983), the follicle walls collapse and become deeply folded with a loss of distinction between granulosa and thecal cells by 24 to 48 h after ovulation (Donaldson & Hansel, 1965a). Mitotic activity was intense in all tissue elements: luteal, stromal, and vascular endothelium and connective tissue trabeculae in the center of the folds were distinct. By day 3 after estrus, folds of the walls met, and by day 4 the cavity was obliterated. Connective tissue trabeculae of thecal externa origin were still apparent.

By day 5-8 after estrus, the corpus luteum becomes more homogeneous. While mitosis of all elements was still high early in this period, they decreased for granulosa luteal cells while there was an increase in the rate of hypertrophy of these cells. Later, large luteal cells were found to be associated with several small luteal cells of thecal origin, a blood vessel and lymphatic duct. Mitosis was confined to the small luteal cells. Toward the end of this period, mitosis was confined to stromal elements and hypertrophy of luteal cells (particularly large luteal cells of granulosa origin) and nuclei was noted.

Corpus luteum growth continued until about day 12, but the CL was fully developed and vasularized by day 9 after ovulation. As the cycle progressed (day 12-18), connective tissue continued to infiltrate the CL and hypertrophy of the blood vessels occurred,
increasing the thickness of vessel walls and in some cases obliterating their lumen.

The period from day 18 through 21 was characterized by degradation of the CL. Connective tissue invasion and hypertrophy of blood vessels continued. The first indications of degradation are a decrease in cytoplasmic stippling and rounding of the cell outlines. This was followed by cell shrinkage, cytoplasmic darkening and nuclear pyknosis with frequent presence of mast cells and phagocytes. The process of degradation or regression was very rapid and was completed by 2 days after estrus of the new estrous cycle.

**Hormonal Control of Follicular Growth**

Follicular development, endocrine secretion and ultimate transformation to luteal tissue is tied intimately to release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. A complete understanding of how LH and FSH stimulate follicular steroidogenesis necessitates some explanation of the two cell theory of estrogen biosynthesis.

The two cell theory was first proposed by Falck (1959), who reported that neither the granulosa or thecal cells of the follicle are independently capable of estrogen biosynthesis. The combined activities of these cell types are required such that thecal cells metabolize C-21 steroids to androstenedione which are subsequently utilized by granulosa cells for production of estrogens. These observations were supported by studies of Lacroix et al. (1974) in the cow. Thecal cells specifically utilize the $\Delta^5$-pathway for conversion of pregnenolone to androstenedione via 17$\alpha$-hydroxy-
pregnenolone and dehydro-epiandrosterone as opposed to the \( \Delta^4 \) pathway. Consistent with the two-cell theory, thecal cells have a very low aromatase enzyme capacity for converting androstenedione to estrogen. However, granulosa cells are highly efficient in aromatizing androstenedione to estradiol, and have a limited ability to metabolize pregnenolone to androgens due to deficiency of C-21 steroid, 17\( \alpha \)hydroxylase. These findings are consistent with those of Falck (1959), and support the theory that thecal cells synthesize androgens via the \( \Delta^5 \) pathway which are then aromatized by granulosa cells for estrogen production (Hansel & Convey, 1983).

Estrogen production by the follicle is linked directly to gonadotrophin stimulation. To complement the two-cell estrogenic model, Armstrong & Dorrington (1977) proposed that both gonadotrophins, LH and FSH, are required for estrogen production. They reported that LH binds specifically to thecal cells and stimulates androgen production by these cells. Granulosa cells then undergo stimulation by FSH to metabolize the thecal androgens to estrogen. A specificity of binding of gonadotrophins to the specific cell types also was observed by Ireland & Roche (1983b). In this study, they observed that at day 17, binding of FSH to granulosa cells and human chorionic gonadotrophin (hCG), an LH-like hormone, to thecal cells was high. Binding of hCG to granulosa cells was very low in comparison. As the period of CL regression approached, specific binding of hCG increased in granulosa cells, indicating a transformation of granulosa cells from follicular to luteal status. Those follicles which had increased binding of hCG in granulosa cells
produced more estrogen in follicular fluid and had larger diameters (Ireland & Roche, 1982a, 1983a, b). These data have led to a model for steroid control of receptor dynamics in the follicle. Richards et al. (1987) theorized that FSH induces LH receptors on granulosa cells, but FSH will not cause receptor synthesis without estrogen to synergize the FSH effect. Therefore, large estrogen synthesizing follicles acquire LH receptors as a result of increased estradiol secretion which is supported by research from Ireland & Roche (1982a, 1983a, b) who found positive correlations between follicular diameter, estradiol (E2) and progesterone (P4) concentrations in follicular fluid and binding of [125I] iodo-hCG to granulosa cells. In contrast, estrogen inactive follicles bound little hCG. So it appears that FSH, in conjunction with estrogen, stimulates synthesis of LH receptors. Increased binding of LH then leads to increased capacity to secrete estrogens (Ireland & Roche, 1982a, 1983a, b).

These data suggest a positive correlation between estrogen production and follicular viability. Henderson et al. (1987) reported that cells from atretic follicles of all sizes produced low amounts of estrogen and high amounts of P4 in comparison to normal non-atretic follicles. These results are supported by other reports (Staigmiller et al., 1982; Ireland & Roche, 1982a, 1983a, b; Tsonis et al., 1984). Aromatase activity was measured in granulosa cells, and found to be higher in non-atretic than atretic follicles (Tsonis et al., 1984). Staigmiller et al. (1982) reported a positive correlation between binding of hCG to thecal cells and estrogen secretion (r=0.68), reflecting receptor populations present on
preovulatory follicles during estrus, when estrogen synthesis is highest (Chenault et al., 1975).

Of recent interest are studies relating follicular fluid concentrations of β-carotene, vitamin E, cholesterol, and vitamin A to follicular function (Schweigert et al., 1987; Schweigert & Zucker, 1988). Concentrations of β-carotene, vitamin E, and cholesterol concentrations in follicular fluid of viable and atretic follicles did not differ, but vitamin A was elevated in follicular fluid of viable follicles (Schweigert & Zucker, 1988). It was hypothesized that β-carotene, vitamin E and cholesterol were transported into follicular fluid from the blood by passive transfer while bound to high density lipoproteins. However, elevated concentrations of vitamin A in follicular fluid, probably represents a metabolic conversion from β-carotene. Vitamin A may then influence follicular development and thereby act as a factor regulating recruitment, selection, and growth of dominant follicles.

**Hormonal Control of the CL**

**Origin of the cell types.** The end result of follicular growth and development is ovulation. This affords an opportunity for fertilization and conceptus development. While the follicle served as a source of nourishment for the ovum, the uterus and oviduct assume this role during early conceptus development. Establishment of a uterine environment conducive to embryonic development is dependent upon P4 secretion from the CL. In effect, the follicle, which becomes the CL, is still serving the role of supporting not only development of the ovum, but the products of conception as well.
The formation of the CL has been described (Donaldson & Hansel, 1965a; Friedkains et al., 1968). The relevance of transformation of the follicle to a CL, as it relates to CL function and development, will now be addressed. In cattle (Donaldson & Hansel, 1965a; Preidkains et al., 1968; Koos & Hansel, 1981; Alila & Hansel, 1984; Fields et al., 1985; Chegini et al., 1984; Alila et al., 1988), sheep (O'Shea et al., 1980; Fitz et al., 1982; Rodgers and O'Shea, 1982; Rodgers et al., 1983a), and other species the CL has been described as containing two distinct, steroidogenic cell populations (large and small luteal cells). It has been hypothesized that small luteal cells are derived from thecal interna and large luteal cells from granulosa cells of the ovulatory follicle. Small luteal cells are 15-18 \( \mu \)m and large cells 18-45 \( \mu \)m in diameter for cattle (Chegini et al., 1984). Similar estimates for small and large luteal cells of sheep were obtained (12-22 \( \mu \)m and 23-25 \( \mu \)m, respectively; Fitz et al., 1982). These cell types differ also in that small luteal cells, during mid-cycle: 1) possess eccentrically located, indented, cup-shaped nuclei with heterochromatin lining the nuclear envelop; 2) lack granules in the cytoplasm; 3) have relatively few microvilli on a relatively smooth surface; 4) contain both smooth and rough endoplasmic reticulum; and 5) possess a large golgi complex and pleomorphic mitochondria with tubular cristae situated in an arc opposite the nucleus. In contrast, the large luteal cells possess: 1) centrally located, round nuclei, with dispersed chromatin and prominent nucleoli; 2) numerous electron dense granules in the cytoplasm; 3) highly convoluted cell surfaces; 4) extensive smooth
endoplasmic reticulum and 5) two types of mitochondria (Koos & Hansel, 1981).

Alila & Hansel (1984) developed specific monoclonal antibodies to granulosa and thecal cells of the bovine follicle. They used these to develop an assay whereby the fate of theca interna and granulosa cells within the CL could be assessed. Percentage of large luteal cells binding granulosa specific antibody at days 4-6, 10-12, and 16-18 were 77 ± 6, 47.5 ± 3, and 30.2 ± 2, respectively. Percentage of small cells bound by granulosa cell specific antibody was 14% on days 4-6 and none were labelled thereafter. When antibody specific to thecal cells was introduced to large luteal cells, binding increased 10 ± 1.3% between days 4-6 and 46 ± 3% between days 10-12. Thecal specific antibody bound a majority of small luteal cells on days 4-6, 10-12, and 16-18 (70 ± 4, 69 ± 3, and 58 ± 6%, respectively).

Binding of granulosa specific antibody to large luteal cells decreased during pregnancy, but binding to thecal specific antibody increased (Alila & Hansel, 1984). These results indicate that small luteal cells and large luteal cells are derived from thecal and granulosa cells, respectively, and that small luteal cells develop into large luteal cells as the CL matures. Some controversy exists as to the validity of the latter part of this hypothesis due to inability of any other laboratory to repeat these results. It represents an eloquent model, but needs to be tested further.

Binding to all cells for either antibody decreased with CL maturity, and it can not be ruled out that the antigenic domains of the luteal tissues recognized by the antibodies might be altered to some degree.
or lost during the transformation from follicular to luteal cells or during maturation of these cells.

**Characteristics of the cell types.** Receptor populations and endocrine secretion by luteal tissue and specific cell types have been characterized in cattle (Spicer et al., 1981; Milvae & Hansel, 1983; Alila et al., 1988) and sheep (Fitz et al., 1982; Rodgers & O'Shea, 1982; Rodgers et al., 1983a; Harrison et al., 1987). In both species, P4 is produced by the small and large luteal cells. Basal secretion rates of P4 are much higher for large luteal cells than small luteal cells (Harrison et al., 1987; Alila et al., 1988). Luteinizing hormone stimulates P4 secretion by small luteal cells but not large luteal cells of both cattle and sheep. These results can be explained based on receptor populations on the luteal cell types. In sheep (Fitz et al., 1982), LH receptor binding sites per cell were greater for small luteal cells than for large luteal cells (33,260 vs. 3,074, during the breeding season). In contrast to these results, Harrison et al. (1987) reported that the number of LH receptors per cell were not different for large or small luteal cells. They also were not different on days 10 or 15 of the estrous cycle. They hypothesized that this discrepancy may be due to the source of luteal cells. That is, Fitz et al. (1982) obtained luteal cells from superovulated CL, from cyclic ewes, whereas Harrison et al. (1987) obtained them following spontaneous ovulations. The effects of PMSG have been reported (Cran, 1983) and described as causing luteinized granulosa cells leading to CL composed of hypertrophied thecal-lutein cells. One consistent result for both
cattle (Alila et al., 1988) and sheep (Fitz et al., 1982; Henderson et al., 1987) is that large luteal cells have higher basal secretion rates of P4 than small luteal cells, and that LH-stimulated P4 secretion is high in small luteal cells while large luteal cells are unresponsive to LH. This probably reflects differences in metabolic activities of the cell types. However, the presence of LH receptors on non-responsive large luteal cells is not consistent with their responsiveness to LH and requires further research.

In support of the theory proposed by Alila & Hansel (1984) that small luteal cells develop into large luteal cells is a study by Chegini et al. (1984). They found that basal, hCG-stimulated, or cyclic AMP-stimulated P4 production; apparent dissociation constants for $[^{125}I]$ hCG binding and total number of available binding sites for hCG on small and large luteal cells during pregnancy were similar. Also, morphological characteristics were more similar than dissimilar for the two cell types during pregnancy. Increasing similarities between the cell types during pregnancy lends additional support to the theory proposed by Alila & Hansel (1984).

Fitz et al. (1982) also quantified prostaglandin F-2α (PGF-2α) and prostaglandin E-2 (PGE-2) receptor numbers on ovine luteal cells. They reported that specific binding sites for both PGF-2α and PGE-2 were approximately 32-fold and 12-fold higher for large than for small luteal cells. Rao et al. (1979) and Bartol et al. (1981) reported that PGF-2α binding to membranes of CL tissue increased from day 3 to day 20 and decreased on days 21-24. Rao et al. (1979) also reported that the relative affinity of PGF-2α binding was low on days
3 and 13 compared to day 20. Relative affinity was actually 203-fold higher on day 20 compared to day 13. Collectively, these data suggest that the CL is less responsive to PGF-2α early compared to late cycle and that large luteal cells respond to PGF-2α more than small luteal cells. This will be of significance in a later discussion of luteolysis.

The responsiveness of large and small luteal cells to PGF-2α has been examined in cattle (Alila et al., 1988). Alila et al. (1988) observed that PGF-2α stimulated P4 production by small luteal cells. This is contrary to the currently accepted theory of the luteolytic mechanism, but the authors hypothesized that the preponderance of small luteal cells early in the estrous cycle might act in a paracrine manner for regulation of P4 production. It has been clearly demonstrated that the CL has the capacity to secrete PGF-2α (Shemesh & Hansel, 1975; Milvae & Hansel, 1983). It has also been shown that the majority of receptors for PGF-2α for luteal tissues of sheep reside on the large luteal cells (Fitz et al., 1982). The relative affinity of PGF-2α for its receptor on the CL is low early in the estrous cycle and increases to the time of luteal regression (Rao et al., 1979). It seems possible that during the early stage of the estrous cycle, when receptor affinities for PGF-2α on large luteal cells are low, PGF-2α could stimulate P4 production by small cells as demonstrated in vitro by Alila et al. (1988).

Luteotrophic substances. Several substances have been described as being luteotrophic. Luteinizing hormone is the classic example of a luteotrophin; a substance which stimulates P4 production by the CL.
Numerous studies have demonstrated that LH and hCG are stimulatory to P4 production in cattle (Schomberg et al., 1967; Milvae et al., 1983) and sheep (Suter et al., 1980). Early studies evaluated the effect of LH or hCG administration on estrous cycle length of cattle (Wiltbank et al., 1961; Donaldson & Hansel, 1965b). They found that LH and hCG extended CL lifespan and increased embryonic survival rates. Estrous cycle extension following treatment with LH or hCG initially may have been ascribed to a direct extension of CL lifespan, but in view of the recent findings of McDermott et al. (1986) a different conclusion may be drawn. McDermott et al. (1986) administered hCG (3,300 IU) to cattle on day 15 after estrus and reported that this treatment regime caused follicular luteinization and extended cycles, due to a reduction in follicular estrogen secretion to initiate uterine PGF-2α secretion. This effect of hCG is probably the same which brought about cycle extension in the earlier studies.

Though LH is widely accepted as having a luteotrophic role, many other substances have also been described as having luteotrophic activities. Among these the catecholamines epinephrine (E; Black & Duby, 1965) and norepinephrine (NE; Auletta et al., 1972) prevent the normal effect of oxytocin induced luteolysis in the cow. Similarly, E, NE and isoproterenol (IPNE) all stimulated P4 production by luteal tissues cultured in vitro from day 8 to 16 of the estrous cycle (Condon & Black, 1976). In this study, they also determined that the catecholamine induced stimulation in P4 production was mediated by beta-adrenergic receptors. Preincubation of luteal tissue with
propranolol (a beta-adrenergic receptor blocker) inhibited catecholamine (E, NE, IPNE) and also gonadotrophic (LH) stimulated P4 production. Condon & Black (1976) also evaluated the effect of phenoxybenzamine (an alpha-adrenergic receptor blocker) on P4 production and found no inhibitory effect of phenoxybenzamine preincubation on E, NE, IPNE, or LH stimulated P4 production. In a separate study, Jordon et al. (1978) evaluated effects of propranolol preincubation on LH stimulated P4 production. They utilized a much lower dose of propranolol than Condon & Black (1976) and found no effect on P4 production by luteal tissue. The exact role of the beta-andrenergic receptor in P4 production by luteal tissues is yet to be fully understood.

In a more recent study, Milvae et al. (1983) again demonstrated catecholamine (E, IPNE) and LH stimulated increases in P4 production by luteal cells. To understand this mechanism further, they tested the effect of a methylation inhibitor (S-adenosylhomocysteine, SAH) on catecholamine and LH stimulated P4 production and found that presence of SAH inhibited E and IPNE stimulated P4 production. Similarly, incubation of tissues with S-adenosylmethionine (SAM, an endogenous stimulator of methylation in membranes) and LH caused an elevation of the stimulatory effect of LH on luteal cell P4 production. Therefore, methylation appears to be involved in the mechanisms whereby LH stimulates luteal cells to produce P4.

Some prostaglandins were ascribed with having a role as luteotrophic agents. As mentioned earlier, PGF-2α has been shown to have a luteotrophic effect on small luteal cells in vitro (Hixon &
The stimulation of P4 production by PGF-2α was similar to that obtained by addition of phorbol ester or phospholipase C. Davis et al. (1987) reported that PGF-2α acts by stimulating phosphatidylinositol 4,5-trisphosphate hydrolysis in the small luteal cells which results in release of diacylglycerol, an activator of protein kinase C. Thus, PGF-2α stimulated P4 production appears to be mediated through protein kinase C activation in small luteal cells.

Several studies have evaluated the role of prostaglandin E-2 in CL function (Chenault, 1983; Gimenez & Henricks, 1983; Reynolds et al., 1983; Chenault et al., 1984). In one study, (Chenault, 1983), it was reported that intrauterine infusion from day 14 to 24 or 28 after estrus of PGE-2 delayed luteolysis, though not for a period of time exceeding the period of infusions. In contrast to these findings, Reynolds et al. (1983) found no extension of cycle length due to intrauterine infusion of PGE-2 alone, but did report extension of CL lifespan for cows receiving PGE-2 and estradiol in combination. They theorized that estradiol-17β and PGE-2, which represent conceptus secretory products, may act synergistically through a luteotrophic mechanism during early pregnancy to maintain luteal function.

Interest in the role of luteal prostacyclin (PGI-2) has increased since Sun et al. (1977) reported that the predominant form of prostaglandin produced by luteal membrane preparations when incubated with PGH-2 (the endoperoxide precursor of prostaglandins for the F, E, D, and I series) was PGI-2. Prostacyclins have been shown to display luteotrophic activities (Milvae, 1986). Injection of PGI-2
directly into the CL on day 10 of the estrous cycle caused elevated P4 concentrations in jugular vein plasma within 5 min (Milvae & Hansel, 1980). Similarly, PGI-2 increased P4 production by dispersed luteal cells in vitro (Milvae & Hansel, 1980). Bovine luteal cells collected on days 5, 10, 15, and 18 after estrus produced 128 ± 12, 87 ± 18, 38 ± 9, and 54 ± 7 ng/10^6 cells of PGI-2, respectively (Milvae & Hansel, 1983), and concentrations of PGI-2 and P4 secretion followed similar trends in this experiment. Of additional interest is that luteal production of PGI-2 was elevated on day 25 of pregnancy compared to day 25 of the cycle (Milvae, 1986). Collectively, these data show relations between PGI-2 and P4 secretion and implicate PGI-2 as having an important luteotrophic role.

**Products of the CL.** The CL has been identified as the source of several hormones, but primarily P4. Progesterone is secreted by large and small luteal cells which are variably responsive to the luteotrophic action of LH (Hansel et al., 1973). A brief description of the P4 secretory pattern of the cow follows. This topic has been extensively reviewed (Henricks et al., 1970; Lemon et al., 1975; Chenault et al., 1975). Briefly, peripheral P4 concentrations are low (less than 0.5 ng/ml) from approximately 2 days preceding to 3 days following ovulation. Following ovulation, luteinization of thecal and granulosa elements ensues. Progesterone concentrations rise from day 4 to about day 12 (Schams et al., 1977). Henricks et al. (1970) reported that P4 concentrations rose 0.73 ng/ml/day and 0.69 ng/ml/day from days 0 to 8 after estrus for pregnant and non-
pregnant cattle and then rose 0.61 and 0.15 ng/ml/day thereafter. This gives some evidence that the conceptus might be mediating luteotrophic mechanisms as its presence was associated with elevated P4 secretion. Peak levels of P4 were reached by day 16 to 18 after estrus and thereafter steadily declined to less than 0.5 ng/ml/day at the next estrus (Henricks et al., 1970).

Recently, oxytocin has been identified as being a product of the CL of cattle (Fields et al., 1983; Wathes et al., 1983; Hansel & Dowd, 1986) and sheep (Rodgers et al., 1983b; Harrison et al., 1987; Schams et al., 1987). Oxytocin production was localized to large luteal cells of sheep, and its secretion was maximal during the early phase of the estrous cycle (Rodgers et al., 1983b; Harrison et al., 1987). Measurement of oxytocin concentrations in the vena cava of cows revealed pulsatile secretory responses which parallel progesterone secretion (Walters & Schallenberger, 1984; Walters et al., 1984). The release of oxytocin also paralleled release of PGF-2α during the period of luteolysis. Thus, oxytocin is not only involved in the luteolytic mechanism of PGF-2α but may also be involved in regulation of P4 secretion early in the estrous cycle (Schams, 1987). Schams (1987) proposed that by regulating P4 secretion early in the estrous cycle, extension of the cycle may occur which is supported by results of others who have shown that P4 supplementation early in the estrous cycle (day 1-5) caused short cycles (Woody et al., 1967; Cinther, 1968, 1969; Lawson & Cahill, 1983). Progesterone supplementation was believed to shorten the estrous cycle by prematurely activating the PGF-2α synthetic
mechanism of the uterus resulting in luteolysis (Baird et al., 1976; Ottobre et al., 1980). Therefore, oxytocin secretion early in the estrous cycle may act to reduce early P4 secretion and thereby extend the functional lifespan of the CL.

Other products of the CL recently identified are GnRH-like proteins (Aten et al., 1987; Ireland et al., 1988). These proteins exhibit potent antigonadotrophic activities thereby suppressing the stimulatory effects of LH. The concentrations of P4 in blood and the capacity of luteal tissues to respond to LH by increasing P4 production have been shown to increase with advancement of the cycle (Milvae & Hansel, 1983). The concentrations of GnRH-like peptides decrease coincidentally in a similar manner (Ireland et al., 1988). It might be hypothesized then that the GnRH-like peptides regulate steroidogenic capacity of the CL early in the cycle possibly allowing longer interestrous intervals than would occur in the absence of GnRH-like peptides. GnRH-like proteins have also been found in granulosa cells of the cow (Ireland et al., 1988) and are actually present in higher concentrations in granulosa than luteal cells. Due to their potent antigonadotrophic activities they may act similarly to regulate growth and atresia of follicles.

**Intraovarian Regulation**

Increasing evidence suggests that intraovarian regulation of follicles occurs. Research directed at elucidating mechanisms whereby a follicle is selected to ovulate has led to studies of follicular dominance. As previously described, follicles undergo recruitment from the dormant pool of primordial follicles in response
to a stimulus, probably FSH. Recruited follicles then develop to the antral stage and selection of one of these to develop to ovulatory size ensues. The follicle selected then suppresses growth of the other follicles until it ovulates or undergoes atresia (Ireland & Roche, 1987). That the largest follicle inhibits growth of lesser follicles was reported by Matton et al. (1981) who, after cauterizing all follicles on the ovaries of heifers, noted rapid proliferation of new follicles to replace them. Follicular factors have been identified which may play a role in establishing dominance of a single follicle on the ovary at any time. The interesting aspect of this theory is that the dominant follicle itself must continue to develop while inhibiting development of other follicles.

One possible regulatory factor which has been identified is inhibin. Henderson & Franchimont (1983) reported that granulosa cells from cattle produce inhibin in vitro. Furthermore, follicular fluid, which contains inhibin-like activity, caused decreased FSH secretion in ovariectomized heifers (Ireland et al., 1983). Therefore, inhibin may act to depress FSH secretion from the anterior pituitary thereby suppressing growth of, or recruitment and selection of, additional dominant follicles (Padmanabhan et al., 1984).

Another intraovarian regulator, follicular regulatory protein (FRP), has been identified in ovarian venous blood (diZerega et al., 1982), follicular fluid (diZerega et al., 1983b) and medium from cultured granulosa cells (diZerega, 1983a) of humans. This compound appeared to inhibit the aromatase activity of granulosa cells in vitro (diZerega & Wilks, 1984). Also, concentrations of FRP of estrogen
inactive (atretic) follicles was twice that of estrogen active follicles (Ireland & Roche, 1987). A model, proposed by Ireland & Roche (1987) to explain the phenomenon of dominance, theorized that the dominant follicle, at any particular moment, secretes inhibin which suppresses pituitary release of FSH which blocks recruitment and selection of new dominant follicles. Then, FRP is secreted by the dominant follicle which impairs the aromatase system of other non-dominant follicles resulting in their atresia. Estradiol secretion by the dominant follicle, in turn, enhances its sensitivity to gonadotrophic stimulation thus ensuring its survival. The GnRH-like peptides described by Aten et al. (1987) and Ireland et al. (1988) may also play a role in this process because these molecules have been shown to possess antigonadotrophic activities, specifically to attenuate LH-induced cyclic adenosine monophosphate (cAMP) accumulation in rat luteal cells. Massicotte et al. (1980) also have shown GnRH or its agonists suppress FSH-induced accumulation of cAMP in porcine granulosa cells. Based on knowledge that GnRH-like peptides are found in granulosa cells and that agonists to GnRH affect FSH stimulation of granulosa cells, it does not seem unlikely that GnRH-like peptides might be involved in control of follicular steroidogenesis and thereby control dominance. Although it is not known at this time, GnRH-like peptides might be secreted by the dominant follicle to suppress other non-dominant follicles.

If a follicle has established itself and gained dominance, it will either ovulate or undergo atresia. If the follicle does not ovulate, FRP continues to accumulate in the follicular fluid of the dominant
follicle, impairing its estrogen producing ability and it eventually undergoes atresia. This relieves the negative inhibition on FSH secretion by the pituitary and results in recruitment of a new set or wave of follicles in response to FSH release.

**Gonadotrophin Secretion during the Bovine Estrous Cycle**

The release pattern of LH from the anterior pituitary has been studied extensively. Generally, LH secretion is low during most of the estrous cycle and becomes elevated for a short period of time immediately following onset of estrus (Henricks et al., 1970; Spicer et al., 1981). Serum P4 and specific binding of hCG to the CL increase from 1.9 to 4.5 days after the preovulatory LH surge, remained unchanged between 8.3 and 12.4 days post-LH surge, and declined thereafter (Spicer et al., 1981) indicating that the CL becomes more responsive to LH concomittant with elevated P4 secretion. Secretory patterns of LH have revealed pulsatile patterns that are dependent upon the steroids being secreted. The early luteal period, day 3, was characterized by low amplitude (LH, 0.3-1.8 ng) and high frequency pulses (16-30 pulses every 24 hours (Rahe et al., 1980; Walters et al., 1984). During the mid-luteal phase of the estrous cycle, days 10 to 11, LH pulses were classified as high amplitude (LH, 1.2-7.0 ng) and low frequency (6-8 pulses every 24 hours). Ireland & Roche (1982b) found that insertion of progesterone-releasing intravaginal devices (PRID) caused decreased pulse frequencies, and the number of pulses increased after PRID removal, which supports the hypothesis that P4 inhibits pulses of
LH, but does not itself affect pulse amplitude (Ireland & Roche, 1982b).

Luteolysis was induced in a group of cattle which resulted in a preovulatory surge of LH 59 h after administration of the prostaglandin analogue, cloprostenol (Walters & Schallenberger, 1984). This pulse of LH was the result of simultaneous increases in the pulse frequency (pulse interval = 38-40 min versus 200 min for mid-luteal period) and amplitude (7-32 ng/ml) of LH just prior to the LH surge. Rahe et al. (1980) also reported increased LH pulse frequencies and amplitude at the time of LH surge and theorized that LH secretion is probably modulated by ovarian steroids.

Estrogen release was correlated positively to LH pulses (Walters & Schallenberger, 1984; Walters et al., 1984) which was not surprising when viewed in light of the previous discussion of follicular steroidogenesis. One product of the follicle is E2, and its secretion is tied directly to gonadotrophic stimulation of the follicle. It is also likely that E2 modulates LH release. Karsh et al. (1983) evaluated the effect of E2 and P4 in ovariectomized ewes and observed that P4 withdrawal increased pulse frequencies and E2 administration caused a further increase in pulse frequencies and decrease in pulse amplitudes. Collectively, these data support the theory that intimate control of LH pulsatile release is a function of steroid regulation of the anterior pituitary.

The high amplitude, low frequency pattern of LH secretion during the luteal phase is thought to be the result of P4 negative feedback on hypothalamic release of gonadotrophin releasing hormone (GnRH;
Knobil, 1980) and sensitivity of the pituitary to GnRH (Padmanabhan et al., 1982). Prior to the preovulatory surge, E2 results in decreased sensitivity of the pituitary to GnRH and reduced LH pulse amplitudes (Kesner & Convey, 1982). Then when E2 secretion is maximal, sensitivity of the pituitary gland to GnRH reaches its maximum resulting in increased frequency, but not amplitude of LH release (Walters & Schallenberger, 1984; Kesner & Convey, 1982; Padmanabhan et al., 1982). Walters & Schallenberger (1984) suggested that negative feedback of E2 on the hypothalamus might explain the endocrine patterns observed during the periovulatory period.

Regulation of FSH secretion is less understood. Generally, FSH secretion is higher than LH during the estrous cycle (Walters et al., 1984; Walters & Schallenberger, 1984). Pulse frequencies for FSH were similar to those of LH during the early luteal phase (8.5 vs. 8.0 pulses every 12 hours; Walters et al., 1984). In contrast to LH, FSH pulse frequencies changed little during the mid-luteal phase (LH = 3.6 vs. FSH = 6.3 pulses every 12 hours). Walters et al. (1984) also found that 90-100% of all LH/FSH and separate FSH pulses were associated with pulses of P4. Pulse amplitude for FSH increased as ovulation approached (Walters & Schallenberger, 1984), and a second surge was reported to occur 4 to 12 h after the LH-surge. This was thought to be due to an increase in amplitude and not frequency of pulses (Walters & Schallenberger, 1984).

Administration of LH or hCG has been shown to profoundly affect luteal function (Wiltbank et al., 1961; Donaldson & Hansel, 1965b). In these studies, LH or hCG administration resulted in prolongation
of CL lifespan and increased embryonic survival. Some of this effect may have been due to the luteotrophic action of LH but other mechanisms have been suggested (Schomberg et al., 1967; McDermott et al., 1986). Schomberg et al. (1967) found that administration of LH or hCG resulted in prolonged CL lifespan but also caused new ovulations resulting in accessory CL. McDermott et al. (1986) also reported formation of accessory CL in response to administration of hCG on day 15 after estrus.

Exogenous administration of hCG early in the estrous cycle has been shown to affect the developing CL and probably not affect follicles as much. Moody & Hansel (1971) reported that administration of 11,000-15,000 IU hCG during days 1 to 7 after estrus increased CL size. Studies in vitro also have indicated that hCG increases P4 content, but not concentration in CL tissue (Moody & Hansel, 1971; Veenhuizen et al., 1972). Therefore, it appears that hCG, given during CL development, causes increased CL size and P4 production, but does not affect the steroidogenic capability of the CL tissue on a per unit mass basis. Helmer & Britt (1987) reported that hCG given on days 2 to 4 did, in fact, increase P4 secretion in heifers given 1000 IU on each day. In contrast, hCG administered during the luteal phase causes cycle extension (Wiltbank et al., 1961; Donaldson & Hansel, 1965b), but this effect was due to new ovulations resulting in formation of accessory CL (Schomberg et al., 1967; McDermott et al., 1986). Thus, hCG seems to have variable effects. Early in the estrous cycle it has luteotrophic effects on the developing CL while in mid-luteal periods it affects
luteinization of follicles and or ovulations resulting in assessor
cL formation and cycle extension.

**Uterine Regulation of CL Lifespan during the Estrous Cycle**

It is generally accepted that the uterus plays an active role in
the luteolytic mechanisms of several species. One of the first
reports of this association was by Loeb (1923) who noted that removal
of the uterus from guinea pigs extended CL function. Surgical
removal of the uteri of both sheep (Wiltbank & Casida, 1956; Anderson
et al., 1969) and cattle (Wiltbank & Casida, 1956; Anderson et al.,
1965) also resulted in extended CL function. The process of uterine-
mediated luteolysis also has been ascribed to being a local rather
than a systemic phenomenon. Ginther et al. (1967) unilaterally
hysterectomized heifers and found that oxytocin- induced CL
regression occurred only when the remaining uterine horn was
ipsilateral to the CL bearing ovary. Regression did not occur when
the remaining uterine horn was contralateral to the CL bearing ovary.
That uterine-mediated luteolysis is a local versus systemic effect
has been unequivocally demonstrated for the cow and ewe (Ginther,

The vasculature of the uterus and ovaries have been studied to
elucidate the local mechanism of luteolysis (Wallmerhaus, 1964;
Passage of the luteolytic substance from uterus to ovary was
theorized to occur via transfer from uterine venous drainage into
ovarian arterial supply by a countercurrent exchange mechanism just
below the ovarian vascular pedicle. This countercurrent,
venoarterial transfer is possible because the ovarian artery follows a convoluted and tortuous path on the surface of the uteroovarian venous drainage, the venous drainage of uterus and ovary, at this point, sharing common vessels. Furthermore, walls of the major vessels are thinner in the region of vеноarterial apposition and connective tissue bundles of the two vessels form a single stratum such that demarcation of the vessels is no longer apparent (Wallmerhaus, 1964). Results of these studies strongly support the idea that the uterus is the source of the luteolytic agent causing demise of the CL and that this occurs by a local vеноarterial transfer in sheep and cattle.

Some evidence exists which would modify the model for luteolysis as described thus far. Abdel Rahim et al. (1984) and Heap et al. (1985) suggested that lymphatic drainage of the uterus plays an integral role in the luteolytic mechanism of sheep. Abdel Rahim et al. (1984) reported that extension of CL lifespan occurred in ewes which had all connections between uterus and ovary severed except the uteroovarian vascular system. Connections between the uterine horn and ipsilateral ovary, i.e. oviduct and accompanying vessels, broad ligament, nerves, lymphatics, and arteries, were all severed. If the uteroovarian countercurrent mechanism was all that was required for luteolysis, this model system should have resulted in normal luteolysis. Since it did not, some additional systems may be required for luteolysis to occur. Heap et al. (1985) supplied evidence that the lymphatic drainage of the uterus plays an integral role in uterine mediated luteolysis by infusion of radiolabelled PGF-
2a into a uterine lymphatic vessel or uterine vein or injection of it into the uterine lumen of anesthetized ewes. When PGF-2α was infused into the uterine lumen of ewes, elevated levels of PGF-2α were observed within 20 min in both uterine lymphatics and uterine venous plasma, but persisted longer in lymphatic secretions. Infusion of PGF-2α into uterine afferent lymphatics resulted in transfer to ovarian artery within 10 min and transfer rates were 0.4% from the lymphatic vessel. This was actually higher than the transfer rate from the uterine vein in this study (0.3%).

It is widely accepted that PGF-2α is the uterine luteolysin of cattle and sheep. This concept is based upon several lines of evidence. Exogenous administration of PGF-2α is luteolytic when administered to cattle (Hansel et al., 1973; Lauderdale, 1974; Thatcher & Chenault, 1976; King et al., 1982). Elevated concentrations of PGF-2α in uterine venous drainage (Nancarrow et al., 1973; Shemesh & Hansel, 1975), uterine tissue (Shemesh & Hansel, 1975), and uterine flushings (Lamothe et al., 1977; Bartol et al., 1981b) are associated with the period of luteal regression. Prostaglandin F-2α was metabolized from arachidonic acid and prostaglandin endoperoxides (prostaglandin H-2) in the uterus and the conversion of PGH-2 to PGF-2α was very efficient (Wlodawer et al., 1976). Concentrations of PGF were also shown to be elevated in the ovarian artery (OA) compared to concentrations in the peripheral circulation (Wolfenson et al., 1985). The difference in concentrations of PGF between ovarian artery and peripheral vein (PV) were highest (OA-PV = 160 pg/ml) during luteal regression (days 19-
Wolfenson et al. (1985) reported that about 1% of the PGF in the uterine venous drainage was transferred to the ovarian vein during luteolysis. Collectively, these results indicate that PGF-2α is the uterine luteolysin in cattle and sheep, since it is produced by and secreted from the uterus during periods corresponding to luteolysis and is shown to have luteolytic properties in vivo.

Although PGF-2α is generally recognized as being the uterine luteolysin, this does not yield insight into how this molecule affects luteolysis. A great deal of effort has gone into the study of the luteolytic mechanism. A working hypothesis incorporates the actions of several ovarian and uterine hormones and their actions on one another. Estradiol has been shown to cause uterine release of PGF-2α and increase the concentration of peripheral PGFM when administered late in the estrous cycle (Barcikowski et al., 1974; Thatcher et al., 1984b; Bartol et al., 1981b; Thatcher et al., 1986b; Hixon & Flint, 1987; Lafrance & Goff, 1988) and result in luteal regression (Wiltbank, 1966; Eley et al., 1979; Thatcher et al., 1986b). However, E2 administration early in the estrous cycle does not cause premature luteolysis (Loy et al., 1960). Furthermore, P4 administration during the first 1 to 5 days after estrus resulted in short estrous cycles (Ginther, 1968; Lawson & Cahill, 1983).

Capacity of the uterus to release PGF-2α is believed to be an effect of P4 priming of the uterus such that after a period of approximately 10 days of P4 exposure, the luteolytic mechanism becomes functional.

Control of susceptibility to E2-induced luteolysis seems to be stage specific and probably dependent upon prior ovarian steroid
modulation. It is known that E2 stimulates synthesis of its own receptors and of P4 receptors (Clark et al., 1977). Furthermore, P4 suppresses estrogen receptor synthesis (Clark et al., 1977) as well as synthesis of its own receptors (Schrader & O'Malley, 1978). Elevated P4 during the luteal phase inhibited formation of E2 receptors on the uterus thereby blocking the luteolytic action of E2 (Henricks & Harris, 1978). Receptors for P4 were also suppressed during the late luteal phase of the estrous cycle presumably by the action of P4 (Zelinski et al., 1982) which would allow E2 from developing follicles to induce E2 receptors, thus making the luteolytic mechanisms operative.

A role for oxytocin in the luteolytic mechanism has also been identified. Administration of exogenous oxytocin has been shown to be luteolytic in cattle (Armstrong & Hansel, 1959; Auletta et al., 1972) by inducing uterine PGF-2α release (Milvae & Hansel, 1980), and elevating peripheral PGFM concentrations (Lafrance & Goff, 1985, 1988). Additionally, it was reported that E2 caused formation of oxytocin receptors following a period of P4 priming in sheep (Roberts et al., 1976; McCraken et al., 1984; Hixon & Flint, 1987). Oxytocin release into the vena cava paralleled P4 secretion during the luteal phase and pulse frequency, but not amplitude, increased from 2.0 to 4.7 pulses every 12 h from days 4 to 11 after estrus (Walters et al., 1984). However, oxytocin concentrations during the periovulatory period were very low or undetectable (Walters & Schallenberger, 1984). Transcription of oxytocin messenger ribonucleic acid (mRNA) in bovine corpora lutea was greatest at days 3-6 of the estrous
cycle, while oxytocin content (ng/g tissue) was greatest on days 11-18 of the estrous cycle (Schams et al., 1987). Oxytocin content of corpora albicantia, early developing CL, and CL of pregnant animals was low (Schams et al., 1987). Of interest is that oxytocin has also been demonstrated in follicular fluid (Schams et al., 1985) and it has been localized to granulosa cells by immunocytochemistry (Kruip et al., 1985). Large luteal cells, which are responsible for luteal oxytocin secretion in sheep (Rodgers et al., 1983b) are derived from granulosa cells (Alila & Hansel, 1984). Collectively, these data indicate that the synthetic capacity of the CL to produce oxytocin originates in the follicle. Oxytocin accumulates in the CL following mRNA transcription early in the estrous cycle, reaches maximal concentrations at the mid-luteal phase (Schams et al., 1987) and then is depleted from luteal tissues around the time of luteolysis and is low after CL regression and during pregnancy.

Collectively, these data led McCracken et al. (1981, 1984) to propose a working hypothesis or model for the luteolytic mechanism of cyclic ewes. After P4 priming has occurred, inhibition of E2 receptor formation is relieved. Estrogens stimulate synthesis of their own receptor which when bound by estrogen results in oxytocin receptor synthesis. Oxytocin from the CL and or pituitary interact with the endometrial receptor which stimulates PGF-2α release. The luteolytic action of PGF-2α is to decrease P4 secretion and PGF-2α is also associated with further oxytocin release by the CL. The second release of oxytocin may then reinforce the secretion of PGF-2α from
the uterus. This cycle would continue until the CL is no longer capable of oxytocin synthesis and secretion.

It has been proposed that one mechanism whereby oxytocin and estrogen stimulates PGF-2α release includes increased turnover of phosphoinositides, a possible, but limited, source of arachidonic acid for PGF-2α synthesis (Flint et al., 1986; Hixon & Flint, 1987). Flint et al. (1986) incubated slices of caruncular endometrium from steroid-treated, ovariectomized ewes with [3H]inositol to radiolabel tissue phosphatidylinositol. Treatment with oxytocin was shown to increase incorporation of [3H]inositol into phosphatidylinositol. Phosphoinositides are normally hydrolysed to inositol phosphates and diacylglycerol, the latter of which can be metabolized to arachidonic acid. Tissue slices preincubated with [3H]phosphatidylinositol had increased incorporation of radiolabel into inositol mono-, bis- and tris-phosphates, the latter being the prevalent form after addition of oxytocin to incubations. Furthermore, 72% of [3H]arachidonyldiacylglycerol was converted to [3H]arachidonic acid by caruncular endometrium. In a similar experiment, caruncular endometrium was incubated with [3H]inositol in the presence of E2 and or oxytocin (Hixon & Flint, 1987). In this study, E2 enhanced the oxytocin-induced increase in phosphoinositide turnover which coincided with PGF-2α release and functional luteolysis in sheep (Hixon & Flint, 1987). These data indicate that PGF-2α synthesis by the uterine endometrium may result from increased phosphoinositide turnover, resulting in liberation of arachidonic acid, the precursor of prostaglandins.
Pharris et al. (1970) hypothesized that PGF-2α mediates its luteolytic effect by constriction of ovarian venous drainage. This would result in decreased nutrient supply to the CL and lead to its demise. McCracken et al. (1971) found that injection of PGF-2α directly into the CL resulted in decreased P4 secretion without any corresponding reduction in ovarian blood flow. However, this did not rule out alteration in intraovarian blood flow. Subsequently, Niswender et al. (1976) measured blood flow to the ovaries of sheep and found that blood flow to the CL-bearing ovary increased as the CL developed, was sustained while P4 was elevated, declined within 4 h of intrauterine infusion of PGF-2α, and was followed 2 h later by lowered peripheral P4 concentrations (Niswender et al., 1976). McCracken et al. (1979) and Einer-Jensen & McCracken (1981) reported that P4 secretion decreased 50% by 1 to 2 h after PGF-2α administration and well before any change in capillary blood flow was detected. It appears unclear as to what role decreased blood flow has on inducing luteolysis. It is most likely associated with structural events associated with luteolysis rather than initiation of the event. Along with functional luteolysis (decline in P4 secretion), PGF-2α also induces structural luteolysis (degradation of the luteal tissue). Murdock (1987) demonstrated that the immune/inflammatory system of sheep is active in the process of luteolysis. They reported that PGF-2α elicited production of a chemoattractant for eosinophiles by luteal tissues. Eosinophiles have been reported to release substances which mediate tissue injury (Gleich & Loegering, 1984). Eosinophile accumulation in ovine luteal
tissue is supported by others (Nett et al., 1976a; McClellan et al., 1977). Lysosomes which appear to play a role in luteolysis of ovine CL (McClellan et al., 1977) might be released in response to PGF-2α and unmask autoimmune sites (possibly covered by sialic acid residues) on luteal cells which could be recognized by preexisting antibodies. This process could elicit an inflammatory reaction resulting in the observed accumulation of eosinophiles. Macrophages have also been implicated in cellular luteolysis. Paavola (1979) reported that autophagy (cellular self-digestion) and heterophagy (removal of cells by macrophages) were active components on luteolysis in the guinea pig. Lysosomal release resulting in autophagy was detected in this study and concurred with results of McClellan (1977) in sheep. In the study by Paavola (1979), macrophages played an integral role in the luteolytic mechanism by removing luteal cell fragments as well as whole luteal cells. Murdock (1987) also reported the appearance of macrophages during luteolysis, but only after the peak of eosinophila.

Another mechanism for the luteolytic action of PGF-2α is that it may antagonize local gonadotrophic support of the CL resulting in luteolysis. It was thought that PGF-2α might bind to the luteal cell membrane and prevent the regulatory unit of adenylate cyclase from activating its catalytic unit thereby suppressing P4 production (Henderson & McNatty, 1975). This theory was supported by evidence that PGF-2α does inhibit LH-induced accumulation of intracellular cAMP in luteal cells (Lahav et al., 1976; Thomas et al., 1978; Hamberger et al., 1979). This antigenadotrophic effect is likely
receptor-mediated as gonadotropins have also been demonstrated to inhibit PGF-2α induced luteolysis (Henderson & McNatty, 1975). It has also been shown by X-ray diffraction that membrane structure of luteal cells is changed by PGF-2α (Buhr et al., 1979; Carlson et al., 1981). The action of PGF-2α might be to either limit accessability of LH to its receptor (through altered membrane structure) or by uncoupling of the regulatory unit of adenylate cyclase of the LH receptor as suggested by Henderson & McNatty (1975). In either case, the effect would be to withdraw LH support and cause luteal regression.

Prostaglandin F-2α induced suppression of P4 contradicts data of others who reported increased P4 secretion by small luteal cells in response to PGF-2α (Alila & Hansel, 1984). It is important to note that in the experiment by Alila & Hansel (1984) enriched small and large luteal cell populations were utilized. Pate & Condon (1984) reported a decrease in P4 synthesis for unseparated bovine luteal cells in response to PGF-2α. These results raise several possibilities for control of CL function by a luteal source of PGF-2α. First, receptors for PGF-2α are present in highest numbers on large luteal cells (Fitz et al., 1982), but receptor affinities are low until the period of luteal regression approaches (Rao et al., 1979). This suggests that the effect noted for small luteal cells could regulate P4 production early in the estrous cycle without initiating luteolysis. As receptors on large cells acquire higher affinities for PGF-2α later in the estrous cycle (Rao et al., 1979), large cells could become responsive to luteolytic action of PGF-2α.
Results of Pate & Condon (1984) also imply that intercommunication occurs between large and small luteal cells of the CL to regulate responsiveness of each other.

Uterine Regulation of CL Lifespan During Pregnancy

The presence of a functional CL with continued P4 secretion is an absolute requirement for pregnancy maintenance in the cow and ewe. It has been reported that P4 concentrations in peripheral circulation are elevated as early as day 10 of pregnancy compared to non-pregnant animals (Henricks et al., 1970, 1971; Lukaszewksa & Hansel, 1980). Although this observation has not been fully supported by observations of others (Folman et al., 1973; Sreenan & Diskin, 1983), it suggests that the conceptus may release or induce a luteotrophic factor which counteracts the luteolytic effect of PGF-2α (Henderson & McNatty, 1975). Although PGE-2 has been shown to extend CL function in cattle under some circumstances (Chenault, 1983; Reynolds et al., 1983), its effects are not consistent. Also, no effect of pregnancy on secretion of PGE-2 from cultured (Curl et al., 1983) or perifused (Gross et al., 1988c) bovine endometrium has been demonstrated. In contrast, intrauterine infusion of PGE-2 to ewes has been shown to extend luteal lifespan (Pratt et al., 1979; Huie et al., 1981; Magness et al., 1981). The CL of sheep also becomes more refractory to luteolytic effects of injected PGF-2α during early pregnancy (Inskeep et al., 1975; Mapletoff et al., 1976; Pratt et al., 1977; Silvia & Niswender, 1984) and this refractoriness to PGF-2α also was reported after PGE-2 administration (Henderson et al., 1977; Pratt et
al., 1977; Reynolds et al., 1981). These data, along with results of Ellinwood et al. (1979) and Silvia et al. (1984) indicating that PGE-2 is elevated in uteroovarian venous plasma of pregnant ewes, suggest that the conceptus may exert its antiluteolytic effect, in part, by secretion of PGE-2 which may act as a luteoprotective substance. Prostaglandin E-2 is not elevated during pregnancy in cattle (Curl et al., 1983), but with suppression of uterine PGF secretion the net effect would be to increase PGE-2:PGF ratios.

In addition to the attenuated response of the CL of pregnancy to luteolytic effects of PGF-2α, transfer of PGF-2α from the ovarian vein to the ovarian artery is reduced during pregnancy resulting in lower PGF-2α concentrations reaching the ovary (Wolfenson et al., 1985). Concentrations of PGFM in peripheral circulation of pregnant cattle (Kindahl et al., 1976; Betteridge et al., 1984; Thatcher et al., 1984b) also were lower. Similarly, PGFM and PGF-2α secretions by endometrial tissue cultured (Thatcher et al., 1984b) or perfused (Gross et al., 1988c) from pregnant cattle were reduced compared to cyclic cows from day 17 after estrus. Collectively, these data indicate that bovine endometrial PGF-2α secretion is suppressed during pregnancy.

Uptake of PGF-2α by uterine veins and ovarian arteries of pregnant cattle also was found to be suppressed (Thatcher et al., 1984b). Although differences were not significant, Lewis et al. (1978) reported a trend for selective retention of PGF-2α in ovarian vein during the comparable period for luteolysis in cyclic ewes. No difference in endometrial production of PGF-2α from cyclic and
pregnant ewes was reported and it therefore seems possible that in sheep, PGF-2α secretion might be altered in a manner different than that for cattle. This was supported by others who found mean PGF-2α in ovarian venous blood to be higher (Wilson et al., 1972; Ellinwood et al., 1979) or not different (Nett et al., 1976b; Silva et al., 1984) between pregnant and cyclic ewes at the time of normal luteolysis. Uterine endometrium from pregnant ewes was found to contain more PGF (Lewis et al., 1977; Ellinwood et al., 1979; Findlay et al., 1983) than comparable cyclic endometrium. Endometrium from pregnant ewes secreted more PGF in vitro than cyclic endometrium (Ellinwood et al., 1979; Findlay et al., 1981). Furthermore, rate of secretion of PGF was higher and more was released toward the luminal versus myometrial side of ovine endometrium utilizing a perifusion device (Lacroix & Kann, 1983). Frequent blood sampling led to a theory that, in sheep, PGF-2α secretion by the uterus is not altered by pregnancy, but its pattern of release from the uterus is altered (Zarco et al., 1984). Results of Zarco et al. (1984) indicate that basal secretion of PGF-2α is elevated, but luteolytic pulses are absent during pregnancy so the that ovary does not receive a luteolytic signal leading to demise of the CL. Lacroix & Kann (1986) also found that PGFM pulses were absent in early pregnant ewes. This attenuation of the luteolytic mechanisms in ewes is, therefore, different from that of cows in which synthesis and secretion of uterine PGF-2α is inhibited. In cattle (Thatcher et al., 1984b; Wolfenson et al., 1985) and sheep (Lacroix & Kann, 1983,1986; Zarco et al., 1984) responsiveness of the uterus to agents which stimulate
PGF-2α release in a lytic pulsatile pattern is attenuated by pregnancy.

The model for luteolysis includes estrogen induction of oxytocin receptor and oxytocin activation of the prostaglandin synthesizing machinery of the endometrium. One of the strongest lines of evidence for involvement of oxytocin in luteolysis is extension of luteal function following passive or active immunization against oxytocin in sheep (Sheldrick et al., 1980; Schams et al., 1983). Additionally, stimulation of PGFM release following oxytocin administration is suppressed during pregnancy in both cattle (Lafrance & Goff, 1985) and sheep (Fairclough et al., 1984). Similarly, PGFM release was attenuated in pregnant cattle compared to cyclic cattle in response to E2 injections (Rico et al., 1981; Thatcher et al., 1984b, 1986b). Kittock & Britt (1977) reported that the luteolytic effect of E2 was decreased in pregnant versus cyclic ewes. More recently, Lacroix & Kann (1986) found that pregnancy completely abolished PGFM pulses in response to estradiol. Collectively, these data indicate that mechanisms for initiating luteolysis are attenuated in both sheep and cattle. However, the exact mechanisms for blocking luteolytic pulses of PGF-2α are slightly different. In cattle, synthesis and secretion of PGF by endometrial tissue is suppressed during pregnancy (Thatcher et al., 1984b) as is transfer of PGF from uterine vein to ovarian artery (Wolfenson et al., 1985). In sheep, synthesis of PGF does not seem to be altered. However, there seems to be enhanced secretion of PGF toward the uterine lumen (Lacroix & Kann, 1983) in association with increased basal secretion of PGF (Zarco et al., 1984) and
attenuation of pulsatile release of PGF by the endometrium (Lacroix & Kann, 1980b). In both species, responses to luteolytic doses of oxytocin and E2 are decreased. It also appears that transport of PGF-2α by the countercurrent exchange system of uterus and ovary may decrease. Collectively, these results indicate that, in some fashion, the conceptus is activating an antiluteolytic mechanism to attenuate PGF-2α secretion and or its potential accessibility to the CL.

**Development of the Bovine Conceptus**

With the above discussion in mind, it seems appropriate now to address the mechanism whereby the conceptus mediates its antiluteolytic effects for CL survival. Understanding of this mechanism begins with knowledge of critical stages in conceptus development.

Fertilization rate does not represent a significant factor accounting for embryonic death or "wastage." Henricks et al. (1971) reported that fertilization rate was 89%, but the proportion of embryos surviving to day 42 after insemination was only 60%. Similarly, first service conception rates were 50 to 55% for heifers (Roche et al., 1977; Wishart et al., 1977) and 52 to 57% for dairy cows (Mawhinney & Roche, 1978). The exact period of development in which the majority of pregnancy wastage occurs has been evaluated (Diskin & Sreenan, 1980; Roche et al., 1981). Diskin & Sreenan (1980) reported that survival rates of conceptuses were 93%, 56%, 66%, and 58% on days 8, 12, 16, and 42, respectively. They concluded
that the majority of early embryonic death occurs between days 8 and 16. Similarly, Roche et al. (1981) evaluated time of conceptus death by recovering conceptuses following slaughter. Percentage of animals pregnant on days 3, 8, 14, 18, and 28 after insemination were 81, 84, 75, 60, and 62, respectively. In concurrence with Diskin & Sreenan (1980), they concluded that 10 to 20% of all ovulated ova are not fertilized. These data demonstrated that the majority of remaining embryonic wastage occurs between day 8 and 18, and indicate that this represents a critical period in conceptus development. Further examination to determine if this wastage was associated with conceptus communication with the uterus to signal pregnancy is warranted.

**Timing of the Conceptus Signal Relative to "Maternal Recognition of Pregnancy"**

It is apparent that the conceptus must make its presence known if successful establishment of pregnancy is to occur. If the conceptus does not signal its presence, luteolytic pulses of PGF-2α are released by the uterus resulting in demise of the CL. Successful establishment of pregnancy involves secretion of nutrients from the uterus for conceptus growth as well as an alteration of the luteolytic mechanism for continued CL function and P4 secretion. This process of events has been referred to as "Maternal Recognition of Pregnancy" (Short, 1969). The majority of research in this area has been directed at understanding mechanisms for extension of CL function, which represents only one aspect of maternal recognition of pregnancy. Several mechanisms for extension of CL function have been
reported. The mechanisms most understood are antiluteolytic-antiPGF, antiluteolytic-luteoprotective and luteotrophic ones (see Thatcher et al., 1986a). Luteotrophic mechanisms are those which increase P4 secretion. Antiluteolytic-luteoprotective mechanisms are those which protect the CL or make it less responsive to luteolytic actions of PGF-2α. Antiluteolytic-antiPGF mechanisms are those mechanisms which attenuate PGF-2α synthesis, secretion and type of release by the uterus. Evidence exists for each of these mechanisms and discussion of these comprise the remainder of this review.

Studies in both cattle and sheep have been carried out to determine if there is a critical time when the conceptus "signals" its presence to the uterus. Moor & Rowson (1966a) found that 65% of all embryo transfers in sheep made on day 12 of the estrous cycle resulted in pregnancies whereas only 12% of the ewes receiving embryos on day 13 became pregnant. The effect of embryo removal from 5th to 15th day of the estrous cycle on interestrus interval was examined (Moor & Rowson, 1966b). The mean interestrus interval for all ewes from which embryos were removed between days 5 and 12 of pregnancy was 18.0 ± 0.3 days. In contrast, 93% of ewes from which embryos were removed on days 13, 14, or 15 had extended cycles (24.5 ± 0.8 days). Collectively, these results indicated that a conceptus must be present in utero on day 12 after estrus if proper signalling is to occur in the ewe.

Similar evidence for critical timing of conceptus-uterine communication in establishment of pregnancy exists for cattle. Betteridge et al. (1984) reported that synchronous transfer (± 1 day)
of bovine conceptuses before day 17 after estrus resulted in successful pregnancies, whereas transfer on day 17 resulted in no pregnancies at day 42. Humblot and Dalla Porta (1984) observed that embryo removal on days 9 or 14 had no effect on interval to return to estrus compared to noninseminated, uterine flushed, cyclic controls. In contrast, conceptus removal on day 16 extended interestrous intervals 4 to 7 days. Similarly, Northey and French (1980) reported a cycle extension in cattle from which conceptuses were removed on days 17, 18, or 19 after estrus, but observed no cycle extension following conceptus removal on days 13 or 15 after estrus. These results indicate that the bovine conceptus, like the ovine conceptus, "signals" its presence in a precise and defined time period. This period, which is critical for maintenance of the CL appears to occur between days 15 and 17 after estrus.

Evidence for conceptus-induced maintenance of the CL has predictably lead to a search for the "signals" responsible for mediating associated events; these being establishment of a uterine environment conducive to conceptus development, alteration of the uterine luteolytic mechanism for CL regression, and continuation of P4 secretion. "Maternal Recognition of Pregnancy" encompasses all of these processes. However, this discussion and the majority of research in this area has been dedicated to specifically understanding the process of CL maintenance. With this in mind a review of putative conceptus signals is in order.

The bovine conceptus has been shown to secrete several steroid hormones between days 13 and 16 after estrus. These include P4,
testosterone and small amounts of E2 (Shemesh et al., 1979). Blood flow to the gravid uterine horn is elevated between days 14 and 18 after estrus (Ford et al., 1979) and the rise may be temporarily associated with changes in P4 to E2 ratios in cattle. Indeed, E2 stimulates uterine blood flow of cyclic cattle (Roman-Ponce et al., 1978, Knickerbocker et al., 1986c). A localized increase in uterine blood flow, induced by estrogen secreted by the conceptus, could increase delivery of nutrients for conceptus growth and development. Therefore, an increase in blood flow to the uterus would in effect dilute PGF-2α in the blood draining the uterus, thereby reducing the likelihood of a luteolytic concentration reaching the ovary.

Knickerbocker et al. (1985) reported that bovine conceptuses in culture, using [3H]-P4 as precursor, produced small amounts of estradiol, estrone, and estriol, in order of prevalence. Eley et al. (1983) was unable to detect conceptus production of estrogens, but this may have due to the low sensitivity of their test system. Due to relatively low secretion rates of estrogens by bovine conceptuses, a definitive role for them is unclear at this time. That estrogens are luteolytic when administered late in the estrous cycle (Wiltbank, 1966; Eley et al., 1979; Thatcher et al., 1986b) is evidence for the importance of having low levels of estrogen present during pregnancy. Estrogens secreted by the conceptus may be rendered inactive by conjugation or act locally to alter endometrial function to favor conceptus survival. Secretion of estrogen by the bovine conceptus is distinct from that of pig conceptuses which secrete large quantities of estrogens (Gadsby et al., 1980), that accumulate in uterine
flushings (Ford et al., 1982; Geisert et al., 1982), and appear to be responsible for initiating maintenance of CL function in this species (Thatcher et al., 1986a).

Other reports have provided evidence for conceptus-mediated conversion of radiolabelled androstenedione (Chenault, 1980; Gadsby et al., 1980; Eley et al., 1983), testosterone (Chenault et al., 1980) and P4 (Knickerbocker et al., 1980; Eley et al., 1983) to an array of 5β-reduced metabolites. The significant point of these reports was that conversion of P4 to 5β-reduced pregnanes was favored. Smaller amounts of androstenedione and 5β-reduced androstanes were also produced, but not to a great extent. The high activity of the 5β-reductase system in conceptus tissue was distinct from that of the uterine endometrium which utilizes the 5α-reductase system (Eley et al., 1983). It was hypothesized that 5β-reduced metabolites might act by stimulating erythropoietic activity (Gorshein & Gardner, 1970), hemoglobin synthesis (Necheles & Rai, 1969) and reduce activity of the uterine myometrium (Kubli-Garfias et al., 1979). Another possible role for high activity of the 5β-reductase system in conceptuses might be to reduce availability of precursors for E2 production by the conceptus which would be luteolytic if released in sufficient amounts (Thatcher et al., 1984a).

Prostaglandin production by the bovine conceptus has also been described (Lewis et al., 1982; Lewis & Waterman, 1983; Shemesh et al., 1979). Bovine conceptuses recovered on day 13, 15, or 16 of pregnancy and cultured for 48 h are capable of producing PGF and PGE-
2, the production of which increases with age of the conceptus (Shemesh et al., 1979). Lewis et al. (1982) also reported PGF-2α, PGE-2, and PGFM production by bovine conceptuses collected on days 16 and 19 after mating and cultured for 24 h. Production of these PGs were greater by day 19 versus day 16 conceptuses. Endometrial slices from day 16 and 19 of pregnancy produced PGF-2α, PGFM and PGE-2, but production was similar for these two days. Endometrial tissues and blastocysts metabolized $34.3 \pm 1.5\%$ and $7.5 \pm 1.6\%$ of $[^3H]$ PGF-2α to $[^3H]$ PGFM, respectively.

To further characterize PG production by the maternal-conceptus unit, Lewis & Waterman (1983) evaluated conversion of $[^3H]$arachidonic acid to PGs in co-cultures of conceptus and endometrial tissues. In this experiment, both conceptus and endometrium cultured alone produced PGFM, PGF-2α, and PGE-2, however, production per mg of tissue weight was very low for endometrium compared blastocysts. Total PG secretion by uterine endometrium would, however, exceed that of the conceptus. When blastocysts were cultured in endometrial conditioned culture medium, incorporation of $[^3H]$ arachidonic acid was not altered. In contrast, co-culture of blastocysts with endometrium resulted in increased metabolism of $[^3H]$ arachidonic acid to PGFM and PGE-2 with decreased metabolism to PGF-2α. This represented alterations in metabolism of arachidonic acid to PG products with no alteration in the proportion of arachidonic acid metabolized by the conceptus. Results indicated that endometrium is capable of metabolizing $[^3H]$ PGF-2α to PGFM and of shifting metabolism of $[^3H]$ arachidonic acid away from $[^3H]$ PGF-2α and towards
[³H] PGE-2. This shift from PGF-2α production to PGE-2 production may represent metabolism of conceptus derived PGF-2α to PGE-2, because metabolism of arachidonic acid by the conceptus to PGF-2, PGE-2 and PGFM was not altered by incubation with endometrial culture supernatants. Only when endometrium and conceptus tissue were incubated together was the array of PGs produced altered. This suggests that the endometrium can regulate amounts and ratios of PGs in the uterine lumen during pregnancy.

In cattle and sheep, PG production by the conceptus is probably minimal compared to the secretory capacity of the entire uterine endometrium. With this in mind, the function of conceptus-derived PGs remains obscure. The effects of pregnancy on endometrial PG production as discussed in this review are more dramatic.

In sheep, endometrial PGF-2α production does not appear to be attenuated. In fact, endometrial production is greater in pregnant versus cyclic ewes (Ellinwood et al., 1979; Findlay et al., 1981). Secretion was preferentially directed toward the uterine lumen rather than the blood stream (Lacroix & Kann, 1983), which would result in uterine accumulation of PGF-2α. The antiluteolytic effect of the conceptus in ewes was to increase basal release of PGF-2α from the uterus and eliminate release of pulses of PGF-2α that would be luteolytic (Zarco, 1984).

Uterine flushings from pregnant cattle (Bartol et al., 1981b) were reported to contain considerable quantities of PGF-2α. This accumulation of PGF-2α could be the result of conceptus-derived PGF-2α (Lewis & Waterman, 1983) or result from decreased transfer of
endometrial PGF-2α out of the uterus as proposed by Thatcher et al. (1984a). Wolfenson et al. (1985) demonstrated that ovarian arterial concentrations of PGF were lower for pregnant than non-pregnant cattle supporting the theory that uterine release of PGF-2 was decreased during pregnancy. Similarly, peripheral concentrations of PGFM in pregnant cattle were attenuated (Kindahl et al., 1976; Betteridge et al., 1984; Thatcher et al., 1984a). These results indicate that PGF production by the endometrium, as reported for endometrial tissue in culture (Thatcher et al., 1984b) or perifused (Gross et al., 1988c), is attenuated.

In sheep, PGE-2 secretion by the uterus is elevated (Ellinwood et al., 1979; Silvia et al., 1984) and attenuates the luteolytic effect of PGF-2α (Henderson et al., 1977; Pratt et al., 1977; Reynolds et al., 1981). In cattle, PGE-2 attenuates the luteolytic effects of PGF-2α only for a short period of time (Chenault, 1983; Reynolds et al., 1983) and is not produced in greater amounts by cultured (Curl et al., 1983) or perifused (Gross et al., 1988c) pregnant endometrium. The reduction of PGF-2α secretion in cattle would cause an increase in the PGE-2:PGF-2α ratio in the circulation. Therefore, PGE-2 may play some role in luteal maintenance and hence embryonic survival in cattle, which is supported by limited cycle extensions following PGE-2 administration (Gimenez & Henricks, 1983; Reynolds et al., 1983).

A luteotrophic substance produced by day 13 to 18 bovine conceptuses has been identified (Hickey & Hansel, 1987). This conceptus product, characterized as a small (<10 kDa), heat-labile,
and lipid soluble molecule, was shown to stimulate P4 synthesis by dispersed bovine luteal cells in vitro and suggests that the conceptus secretes luteotrophic signals, to enhance luteal P4 production, prior to secreting the antiluteolytic signal. The nature of this molecule has not been determined, but it was speculated to be either a steroid, luteotrophic prostaglandin, conceptus derived platelet activating factor (PAF) or some combination of these.

Plante et al. (1987) demonstrated that conceptuses produce luteotrophic substances. Trophoblastic tissue from bovine conceptuses were cultured and supernatants tested for luteotrophic activity by culturing with rat granulosa cells. Supernatants increased P4 secretion indicating that luteotrophic substances were secreted by bovine conceptuses.

Although the luteotrophic factor described by Hickey & Hansel (1987) has not been identified, it is not proteinaceous and increasing evidence implicates PAF as a possible candidate for this role.

Conceptuses also have been shown to secrete a variety of proteins, the array of which is age dependent (cattle: Bartol et al., 1981b, 1985; Geisert et al., 1988; Godkin et al., 1988; and sheep: Godkin et al., 1982). Godkin et al. (1982) characterized secretory proteins produced by ovine conceptuses collected on of days 13, 14, 21, and 23 of gestation and cultured for 24 h. They noted on day 13, a time just following the critical period for maternal recognition of pregnancy, that the conceptus produced one major protein. This major
secretory component had a low molecular weight, migrated as an acidic protein (pI = 5.5) and was initially called protein X.

Bartol et al. (1985) characterized bovine conceptus secretory proteins (bCSP) released into medium by days 16, 19, 22, and 24 bovine conceptuses. On day 16, one group of proteins, low molecular weight (22 to 26 kDa) with an isoelectric point of 6.5 to 5.6, represented the major secretory component of bCSP. These proteins may be critical to maintenance of the CL and will be discussed in depth later in this review. Other less prominent polypeptides were present at this stage and as age of the conceptus increased, the distribution of proteins became more complex. The lower molecular weight, acidic proteins increased in abundance by day 19 and declined to days 24 and 29.

More recently, Godkin et al. (1988) characterized bovine conceptus secretory proteins from day 17 to day 38 of pregnancy. Conceptuses were shown to secrete a major low molecular weight acidic protein that represented the major product from days 17 to 22 of pregnancy. In this study, these low molecular weight, acidic proteins were still detectable in trace quantities on day 38. Geisert et al. (1988) collected bovine conceptuses on days 15, 16, and 17 and classified them by length. The low molecular weight acidic proteins were detectable in small quantities from conceptuses averaging 14 mm in length and then secretion increased as conceptus size increased from 40 to 100 mm and greater than 100 mm in length. Complexity of the secretory pattern of proteins increased greatly in conceptuses greater than 100 mm in length.
Protein production by cultured bovine conceptuses (\(\mu\)g/mg wet weight) increased significantly from day 16.5 (4.9 ± 2.4) to day 17.0 (15.8 ± 5.2; Knickerbocker et al., 1986b). These alterations in protein synthetic capacity from day 16.5 to 17.0 correspond to the time of maternal recognition of pregnancy in cows. Results of these studies led to a further examination of effects of pregnancy-specific proteins of conceptus origin on endometrial protein production and function.

The major, low molecular weight acidic protein of day 14-16 ovine conceptuses (hereafter referred to as oTP-1) was purified by Godkin et al. (1982) and its effect on endometrial protein production evaluated (Godkin et al., 1982; Vallet et al., 1987; Salamonsen et al., 1988). Incubation of endometrial tissue from day 12 nonpregnant ewes, with oTP-1 increased incorporation of \([{}^3\text{H}]\)leucine into secreted macromolecules (28 to 48%) and decreased incorporation of radiolabel into tissue proteins (4 to 17%) compared to endometrium incubated with BSA (Godkin et al., 1984a). Specifically, oTP-1 caused enhancement or induction of 6 proteins from culture of endometrium. More recently, Vallet et al., (1987) performed a similar experiment in which day 12 cyclic endometrium was incubated with oTP-1 or BSA. Although percent incorporation of either \([{}^3\text{H}]\) or \([{}^{35}\text{S}]\) methionine into secreted macromolecules was unaffected by treatment, incubation of endometrium with oTP-1 selectively increased secretion of 11 proteins and decreased secretion of 6 proteins compared to BSA-treated tissues. The most striking amplification was of a 70 kDa protein (\(pI = 4\)) whose secretion was increased 370% by incubation.
with oTP-1. Salamonson et al. (1988) also demonstrated that oTP-1 selectively stimulated secretion of 5 proteins by dispersed endometrial cells from sheep.

Bartol et al. (1981b) characterized proteins present in uterine flushings of cyclic and pregnant cattle on days 8 to 19. Total recoverable protein tended to increase toward the end of the estrous cycle (days 14, 16, 19) in cyclic cattle, but there was no significant effect of day on recoverable protein during pregnancy. Generally, recoverable protein was lower in pregnant versus cyclic cattle from days 8 through 16 but amounts were comparable on day 19. The array of proteins recovered on day 19 of the cycle and pregnancy also was analysed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Generally, protein patterns were similar on days 8 through 16 for pregnant and cyclic cattle. However, on day 19 four protein species (relative molecular weights \( \times 10^{-3} = 15.2, 306.8, 322.2, \) and 342.8) specific to uterine flushings from pregnant cattle were identified. Results from this study are difficult to interpret because uterine proteins in uterine flushings may be from endometrium or the conceptus. Knickerbocker et al. (1986b) found that conceptus wet weight and protein synthetic capacity increase significantly on day 17 of pregnancy. Thus, it would appear that presence of the conceptus prior to day 19 decreased protein production by the uterus since total recoverable protein was the same or lower.

Geisert et al. (1988) characterized the qualitative array of proteins secreted into medium after culture of endometrial tissue of
day 17 non-pregnant and pregnant cattle. Induction of a group of low
molecular weight peptides (M_r = 14-16,000; pI = 7.2-6.8) and a
polypeptide with a M_r = 35,000 (pI = 8.4-7.3) by endometrium from the
uterine horn ipsilateral to the CL during pregnancy was observed.
More recently, Gross et al. (1988a) incubated endometrium from day 17
cyclic cattle with bCSP to assess effects upon protein and PG
synthesis. They reported that bCSP decreased incorporation of [^3H]
leucine into both secreted and tissue proteins of endometrial
explants compared to BSA treated controls. While secretion of
protein into medium was decreased by bCSP, secretion of two proteins
(10 and 13 kDa) was amplified.

Presence of a conceptus in utero or intrauterine infusion of
secretory components of the conceptus in cattle and sheep have a
profound effect on endometrial protein synthesis and secretion. The
role of these proteins, whether for alteration of PG metabolism or
for establishing an optimal environment for the developing conceptus
requires further study.

Embryonic homogenates (1 to 2 embryo equivalents/infusion) of day
14-15 ovine conceptuses when infused daily into uterine lumen of
nonpregnant sheep beginning on day 12, extend CL function; estrous
cycle lengths were greater than 22.4 days for ewes receiving
homogenates compared to 16.6 days for controls (Rowson & Moor, 1967).
Daily infusions of day 25 ovine conceptus homogenates, day 14 pig
homogenates or day 14-15 heat treated ovine conceptuses did not
extend the interestrous interval. These results reaffirm that the
antiluteolytic, conceptus signal is stage and species specific and
was possibly proteinaceous in nature. Martal et al. (1979) reported extension of CL function by intrauterine infusion of day 14-16 ovine conceptus homogenates and termed the antiluteolytic substance of the conceptus "Trofoblastin". They found the signal to be stage-specific, as day 21-23 conceptus homogenates would not extend cycles. The signal molecule was heat labile and inactivated by pronase.

As previously described, Northey and French (1980) determined that the bovine conceptus must be present in utero by day 15 to 17 after estrus for establishment of pregnancy to occur. To determine the nature of the conceptus signal, they carried out intrauterine infusions of one homogenized day 17 to 18 bovine conceptus twice daily between days 14 and 18 after estrus in cycling heifers. This treatment regime resulted in an extension of CL function and interestrous intervals (control vs. conceptus homogenate; 21.1 ± 0.74 verus 24.0 ± 0.38 days). Although, infusion of conceptus homogenates extended CL lifespan, there was no evidence of luteotrophic stimulation since concentrations of P4 in serum did not differ between treatment groups. The cycle extension observed in this and other similar experiments is somewhat surprising. Bartol et al. (1985) demonstrated by 2-dimensional polyacrylamide gel electrophoresis that typical bCSP were not easily detected in tissue homogenates of day 22 conceptuses while bCSP were actively secreted into culture medium at this same time. Therefore, it seems likely that conceptus homogenates utilized in the experiments described would contain fewer of the typical, conceptus secretory proteins, yet cycles were extended by 3 to 4 days. In a similar
experiment, Humblot and Dalla Porta (1984) introduced into the uterus whole day 12 conceptuses (2 per infusion) or day 16 conceptuses (1 per infusion), which had been frozen and thawed, transcervically to evaluate their effect on CL lifespan. Day 16 but not day 12 conceptuses extended CL lifespan based on examination of P4 secretory profiles. The interestrous interval for heifers receiving no treatment, or intrauterine infusion of saline, day 12 or day 16 conceptuses twice daily from days 15 to 19 were 22.3, 19.0, 20.0, and 27.0 ± 1.0 days, respectively. Administration of day 16 conceptuses extended interestrous intervals 4 to 7 days. That day 12 conceptuses did not extend interestrous intervals lends support to the theory that the antiluteolytic conceptus signal is stage specific.

The ovine and bovine conceptus "signals" were later shown to be secreted by the trophoblast rather than the inner cell mass or embryo proper (Heyman et al., 1984). Trophoblastic vesicles, which had been derived from sections of day 14 cow and days 11-13 sheep conceptuses cultured for 24 h in vitro, were transferred to recipient cattle and sheep, respectively, on day 12 after estrus. In cattle, trophoblastic vesicles caused extension of cycle length in 8 of 12 recipients which had 25 to 37 day estrous cycles. In sheep, trophoblastic vesicles caused extension of estrous cycles in 7 of 12 recipients, which had 20 to 54 day estrous cycles. Interestrous intervals were extended for 2 of 11 ewes receiving 2 trophoblastic vesicles on day 12 after estrus from day 13 bovine conceptuses (Martal et al., 1984). Similarly, interestrous intervals were extended in 2 of 10 heifers receiving 2 trophoblastic vesicles on day
12 of the estrous cycle from day 11 to 13 ovine conceptuses. This interspecies transfer resulted in extended estrous cycles in about 20% of the cases studied (Martal et al., 1984) compared to 60% extended cycles for intraspecies transfers (Heyman et al., 1984). That interspecies transfers of trophoblastic vesicles is partly effective in extending CL lifespan which implies that the antiluteolytic conceptus signals for these species may be similar.

Data from the preceding studies indicated that the antiluteolytic conceptus signal for the ewe is proteinaceous in nature. The theory that the antiluteolytic signal is proteinaceous was further tested by Godkin et al. (1984b) who infused ovine conceptus secretory proteins (oCSP) and purified oTP-1, which represents the major, low molecular weight (17 kDa) acidic (pI = 5.5) protein produced by day 16 ovine conceptuses and described by Godkin et al., (1982), into the uterine lumen of cyclic day 12 ewes. Intrauterine infusion of oCSP from days 12 to 18 resulted in extension of CL function to days 24, 34, and beyond day 52 in treated ewes. Treatment with oTP-1 extended CL function 4 days longer than controls. These results were confirmed and extended by Vallet et al. (1988b) who infused oCSP from day 16 conceptuses, oTP-1, oCSP minus oTP-1, or serum proteins into cyclic ewes from days 12 to 14. Intrauterine infusion of oCSP or oTP-1 extended the interestrus interval to 25 and 27 days, respectively, compared to control ewes (19 days) and ewes receiving oCSP-oTP-1 (19 days). Ewes in this experiment received injection of E2 on day 14 and oxytocin of day 15 to test uterine response following infusions which may have reduced cycle extension by oTP-1 compared to the cycle
extension which may have occurred without oxytocin and E2. These studies indicate that oTP-1 is the major antiluteolytic agent in oCSP as cycle length was unaffected by infusion of oCSP from which oTP-1 had been removed.

Proteins secreted by days 16 to 18 bovine conceptuses also extended interestrous intervals of cattle receiving intrauterine infusions from days 15 to 21 after estrus (Knickerbocker et al., 1986b). The total complex of bCSP secreted into medium from cultured conceptuses extended interestrous intervals 33.4 ± 2.5 versus 23.5 ± 0.5 days for controls receiving serum proteins. Intrauterine infusion of the primary 5β-reduced steroid from bovine conceptuses, 5β-pregnan-3α-ol-20-one, had no effect on estrous cycle lengths. Collectively, these studies indicate that the major, conceptus derived, antiluteolytic signal responsible for extending CL function is proteinaceous in nature.

Conceptus proteins have also been shown to alter uterine PG secretion in vivo. Release of PGF from the uterus (Vallet et al., 1988b) and peripheral PGFM concentrations (Fincher et al., 1986; Vallet et al., 1988b) in response to E2 or oxytocin was evaluated in cyclic ewes receiving oCSP or oTP-1 on days 12 to 14 of the cycle. In both studies the release of PGF and PGFM was decreased in response to estradiol and oxytocin challenges. In cattle, Knickerbocker et al. (1986b) reported that PGF release by cattle receiving intrauterine infusion of bCSP from days 15 to 18 was attenuated on days 18, 19 and 20 compared to controls receiving serum proteins. Release of PGFM also was decreased in cattle receiving bCSP infusions
from day 15.5 and injected with E2 on day 18 after estrus (Knickerbocker et al., 1986a).

A component of bCSP and oCSP (oTP-1 in sheep) appears to be the antiluteolytic signal of bovine and ovine conceptuses. One remaining question is, how are these proteins acting to decrease uterine PG synthesis and or secretion? One possibility is that conceptus antiluteolytic signals stimulate induction of an endometrial intracellular inhibitor of enzymes involved in PG synthesis. An inhibitor of PG synthesis was identified in uterine preparations of cyclic cows by Wlodawer et al. (1976). This inhibiting factor was found to suppress the fatty acid cyclooxygenase system. Shemesh et al. (1984) also found an endogenous, heat-labile, inhibitor of PG synthesis in maternal caruncular tissue of placemtones that modulated placental PG synthesis during pregnancy. Basu and Kindahl (1987) demonstrated the existence of an endogenous endometrial inhibitor of PG synthesis during the bovine estrous cycle and reported that its activity increased during early pregnancy. They also reported that presence of a conceptus increased activity of the inhibitor in the non-gravid uterine horn, suggesting the existence of a humoral factor which regulates PG synthesis rather than the physical presence of the conceptus. However, the theory that the PG synthesis regulator is a humoral-factor should be viewed with caution as it is possible that conceptus products might be carried to the non-gravid uterine horn through the uterine body. Recently, Gross et al. (1988b) demonstrated the presence of an intracellular endometrial inhibitor of PG synthesis which was, again, more active in tissues from
pregnant than cyclic cattle. The inhibitor was in the high speed cytosolic supernatant fraction of endometrial tissues and was proteinaceous.

To determine if conceptus proteins were responsible for induction of the intracellular PG synthesis inhibitor, Gross et al. (1988a) evaluated effects of incubating day 17 cyclic endometrial explants with bCSP, compared to BSA as a control, on induction of the inhibitor. Cytosol from endometrium incubated with bCSP reduced PG synthesis by the cotyledonary microsomal PG generating system (Gross et al., 1988a).

These data indicate that the antiluteolytic agent of bovine conceptuses is proteinaceous, a component of bCSP, and likely acts by inducing an endometrial inhibitor of PG synthesis. As oTP-1 has been identified as the ovine conceptus signal and there seems to be similarities between antiluteolytic mechanisms of ovine and bovine conceptuses, it seems possible that analogous conceptus protein systems may exist.

A great deal is known about the ovine conceptus antiluteolytic signal. Rowson & Moor (1967) first proposed that the conceptus antiluteolytic signal might be proteinaceous in nature since heat-treatment of conceptus homogenates prior to intrauterine infusions abolished their antiluteolytic effect. This was confirmed later by Martal et al. (1979) who termed the putative substance "Trophoblastin". Electrophoretic analysis of oCSP confirmed the presence of a major conceptus secretory product (19 kDa; pI = 5.3-5.7) which was referred to as oTP-1 (Godkin et al., 1982). This
conceptus signal was demonstrated to attenuate luteolytic PGF release by the uterus (Godkin et al., 1984b; Vallet et al., 1988b) and PGF release in response to E2 and oxytocin administration (Fincher et al., 1986; Vallet et al., 1988b) in cyclic ewes. The source of oTP-1 was identified as the trophectoderm as trophoblastic vesicles will extend CL function in cyclic ewes (Heyman et al., 1984). Godkin et al. (1984a) demonstrated that oTP-1 is taken up by endometrial surface epithelium and superficial glandular epithelium presumably by binding to specific receptors. The antiluteolytic mechanism is not the result of cAMP, cGMP or inositol phospholipid turnover in endometrial tissue (Vallet et al., 1987). Further characterization of oTP-1 demonstrated that three isomeric forms exist and that none are glycosylated (Anthony et al., 1988). In addition to its antiluteolytic effects, oTP-1 has been shown to induce or enhance secretion of several endometrial proteins (Godkin et al., 1982; Vallet et al., 1987; Salamonsen et al., 1988). Hence, oTP-1 appears to be the antiluteolytic signal from the ovine conceptus responsible for initiating events associated with maintenance of CL function. Of particular importance to this review are similarities between the antiluteolytic mechanism for the ewe and cow.

Evidence now exists suggesting that oTP-1 is an interferon-like molecule (Imakowa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988). In these studies, the nucleotide sequence of oTP-1 was determined and the inferred primary amino acid sequence was found to have 45-55% homology with a variety of interferons (IFN) of the alpha
family. The greatest degree of sequence homology was with bovine IFN-α-II (70.3%).

Interferons are molecules which are classically ascribed as having antiviral activity. Three major classes of interferons have been described. These are 1) leukocyte or alpha IFN, 2) fibroblast or beta IFN, and 3) immune or gamma IFN (Pestka & Baron, 1981). As the inferred primary amino acid sequence of oTP-1 indicates homology to the alpha IFN family, the remaining discussion will be limited to this class of IFN. The genes coding for bovine alpha IFN (IFN-α) can be divided into two classes of molecules, class I (IFN-α-I) and class II (IFN-α-II; Capon et al., 1985). Activites for IFN other than their antiviral activities have also been identified, some of which include, antigrowth activity, stimulation of cytotoxic activites of lymphocytes and macrophages and of natural killer cell activity as well as increased expression of some tumor-associated antigens (see Pestka et al., 1987).

Of particular interest to this discussion are IFN effects on PG synthesis. Alpha IFN's have been shown to suppress PGE-2 secretion by mouse monocyte-macrophage (Boraschi et al., 1985) and human mononuclear leukocytes (Dore-Duffy et al., 1983). However, treatment of human leukocytes with IFN-α had no effect on release of PGF (Dore-Duffy et al., 1983). In contrast, Salamonsen et al. (1988) reported suppression of both PGE and PGF-2α secretion of ovine endometrial cells treated with oTP-1 or IFN-α-II. Treatment with IFN-α-I also resulted in extension of interestrous intervals from 22.8 ± 0.8 to 26.8 ± 1.4 days for cyclic cows receiving infusions from days 15.5 to
21 after estrus (Plante et al., 1987). However, in vitro effects of IFN-α-I are in contrast to effects of bCSP. Treatment with bCSP decreased PGF secretion by endometrial explants, induced an intracellular inhibitor(s) of PG synthesis but had no effect on PGE-2 secretion (Gross et al., 1988a). IFN-α-I had no effect on PGF secretion, did not induce the intracellular inhibitor of PG synthesis and enhanced PGE-2 secretion (Plante et al., 1988). The differences may be ascribed to the IFN used in these experiments, that being IFN-α-I, which is not as homologous to the inferred primary sequence of oTP-1 as that of IFN-α-II (Stewart et al., 1987; Imakawa et al., 1987; Charpigny et al., 1988) which was used by Salamonsen et al. (1988).

It seems clear that oTP-1 from the ovine conceptus and some component of bovine conceptuses mediate antiluteolytic regulatory mechanisms in the uterine endometrium. It also is apparent that the regulatory system of the ewe and cow are similar in many respects. In view of these findings, identification, characterization and function of the putative bovine antiluteolytic signal of the conceptus became the objective of research contained in this dissertation.
CHAPTER 2
IDENTIFICATION OF BOVINE TROPHOPLAST PROTEIN-1, A SECRETORY PROTEIN IMMUNOLOGICALLY RELATED TO OVINE TROPHOBLAST PROTEIN-1

Introduction

Embryo transfer experiments have established that maternal recognition of pregnancy in the ewe occurs 12-13 days after onset of estrus (Moor & Rowson, 1966a,b). The corresponding period for the cow is day 15-16 (Betteridge et al., 1984; Northey & French, 1980). In both species the estrous cycle can be extended significantly if extracts of conceptuses (Rowson & Moor, 1967; Martal et al., 1979; Northey & French, 1980; Humblot & Dalla Porta, 1984) or trophoblast tissue (Martial et al., 1984; Heyman et al., 1984) are introduced into the uteri of nonpregnant recipients just before this critical period during which maternal recognition of pregnancy occurs. Evidence has accumulated that in sheep the active substance is proteinaceous and produced for a limited period, not extending beyond days 21-23 of pregnancy (Rowson & Moor, 1967; Martal et al., 1979; Godkin et al., 1982).

Attention has focused on one particular secretory protein of the sheep conceptus, oTP-1 (Godkin et al., 1984a,b; Hansen et al., 1985). This polypeptide is released as a major product by cultured sheep conceptuses between days 13 and 21 and is produced maximally around days 15-17 (Godkin et al., 1982; Hansen et al., 1985). It causes
extension of luteal lifespan when introduced in purified form into uteri of nonpregnant recipient ewes (Godkin et al., 1984b; Vallet et al., 1988b). The protein consists of a group of 3-4 isoelectric variants (pI 5.4-5.7) of relative molecular weight 19,000 (Godkin et al., 1982). Evidence has been presented to indicate that oTP-1 is a hormone-like substance which acts, in a paracrine manner, on the maternal endometrium (Godkin et al., 1984a).

The conceptuses of cows cultured in vitro also release a group of acidic, low molecular weight polypeptides. As with oTP-1, their synthesis is limited to a short, 7-10 day period (days 16-26) which coincides with the time at which maternal recognition of pregnancy occurs (Bartol et al., 1985). Introduction of total unfractonated proteins released by cultured bovine conceptuses into nonpregnant recipient cows causes a significant extension of the interestrous interval (Knickerbocker et al., 1986b). Since the low molecular weight acidic proteins are the major component of bCSP, Bartol et al. (1985) suggested that they may be homologous to oTP-1. In addition, Heyman et al. (1984) have shown that transfer of trophoblastic vesicles, comprised of trophectoderm and extraembryonic endoderm derived from day 11-13 sheep conceptuses, to day 12 recipient cows resulted in extension of luteal lifespan in a significant number of animals. They further demonstrated that reciprocal interspecies transfer of bovine trophoblastic tissue to recipient ewes had a similar effect. Together these results suggest that the trophoblast of ewes and cows produces a functionally similar substance(s) which is recognized by the respective species. The object of the
experiments described in this chapter was to determine whether oTP-1 crossreacts immunologically with component(s) of the bCSP.

Materials and Methods

Materials

All materials used were supplied by vendors as noted by Godkin et al. (1984a), Bartol et al. (1985) and Hansen et al. (1985).

Animals

Adult crossbred ewes, primarily of Rambouillet breeding, were checked twice daily for estrus with vasectomized rams. Ewes were mated at behavioral estrus (day 0) and every 8 to 12 h thereafter to two intact rams at each breeding period. Pregnant ewes were anaesthetized and reproductive tracts were exposed by midventral laparotomy. Intact conceptuses were flushed from uteri on day 16 of pregnancy with a modified minimum essential medium (MEM, see p. 69) at 37°C and collected in sterile serum bottles (Godkin et al., 1982).

Cows and heifers of Holstein, Jersey and crossbred beef breeding from the University of Florida research herds were utilized for collection of bovine conceptuses. The cattle were maintained on pasture and checked for estrus by using visual observation and bulls with penile shunts. All animals were mated at first observation of estrus (day 0) to an intact Brown Swiss bull and artificially inseminated about 12 h later.

Cows and heifers were slaughtered on day 17 or 18 of pregnancy. Reproductive tracts recovered after exsanguination were placed in plastic bags and transported on ice to a sterile, laminar flow hood.
where they were trimmed of excess tissue, including the ovaries and oviducts. The cervix was closed by applying a large, curved, Rochester-Ochsner forcep to the anterior cervix. A plastic, 50-ml syringe fitted with a 16-guage needle was then used to inject 40 ml sterile MEM into the uterine lumen through the tip of the uterine horn contralateral to the ovary bearing the corpus luteum. The anterior tip (about 1 cm) of the horn ipsilateral to the corpus luteum had been removed to provide an enlarged opening. Conceptuses were flushed through this opening into sterile, plastic, culture dishes.

Medium Preparation

Eagle's minimum essential medium (MEM, Gibco custom formula #86-5007) was modified by supplementation with penicillin (100 units/ml), amphotericin B (250 ng/ml), streptomycin (100 µg/ml), insulin (0.2 units/ml), non-essential amino acids (1%, v/v) and glucose (5 mg/ml). Medium also was supplemented with D-Ca pantothenate (100 µg/ml), choline chloride (100 µg/ml), folic acid (100 µg/ml), i-inositol (200 µg/ml), nicotinamide (100 µg/ml), pyridoxal-HCl (100 µg/ml), riboflavin (10 µg/ml) and thiamine (100 µg/ml). Content of leucine was limited to 0.1 times normal (5.15 mg/l) to enhance uptake of L-[³H]leucine when added to cultures. Medium was filter sterilized (0.22 µm) and stored at 4°C.

In-Vitro Culture of Conceptuses

After collection, conceptuses from cows and ewes were transferred to plastic culture dishes containing 15 ml fresh modified (leucine deficient), Eagle's MEM and 100 µCi L-[³H]leucine. Conceptuses
were cultured for 24 h in a controlled atmosphere chamber (Model number 7741-10010, Belco Biological Glassware, Vineland, NJ, U.S.A.), flushed for 10 min with 50% N₂ : 47.5% O₂ : 2.5% CO₂ (v/v) and maintained at 37°C in the dark on a rocking platform. Incubations were terminated by removing conceptuses from culture medium. The medium was frozen at -20°C until used in subsequent studies.

**Purification of oTP-1**

Ovine TP-1 was purified by the method of Godkin et al. (1982). Purity was confirmed by polyacrylamide gel electrophoresis (Godkin et al., 1982).

**Preparation of Conceptus Culture Medium for Analysis**

Medium from cultured conceptuses was centrifuged at 12,000 g for 10 min to sediment cellular material. Supernatant fractions pooled from several cultures then were dialysed (Mₑ cutoff 1,000) extensively (4 liters, changed 4 times) against 10 mM Tris-HCl buffer (pH 8.2) at 4°C to remove low molecular weight compounds, e.g. salts and unincorporated radiolabelled precursors. Whenever the protein concentration of a sample was too low for immediate analysis, samples were dialysed (Mₑ cutoff 1,000) against double distilled water, lyophilized and resolubilized in an appropriate buffer.

**Protein Determination**

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.
Immunodiffusion Techniques

Immunodiffusion plates containing 5 ml 1% (w/v) agarose in 0.07 M sodium phosphate buffer (pH 7.4) were prepared using 6-well tissue culture plates (diameter 35 mm) according to the method of Ouchterlony & Nilsson (1974). Antibody specificity was determined by placing antiserum raised in rabbits against oTP-1 (Godkin et al., 1984a) in the center well of each plate with various dilutions of ovine CSP and bovine CSP introduced into the outer wells. The plates were placed in a container to maintain a moist atmosphere. Resultant precipitation bands were observed at 24-48 h at room temperature. Plates were extensively washed in Dulbecco's saline (PBS; 0.142 g KCl/l; 2.16 g KH₂PO₄·7 H₂O/l, 8 g NaCl/l; Dulbecco & Vogt, 1954) to elute nonprecipitated material, and the bands stained with Coomassie blue. After destaining, gels were photographed.

Solid-Phase Radiobinding Assay

Bovine conceptus secretory proteins, purified oTP-1 and bovine serum albumin were each adjusted to a concentration of 50 μg/ml in PBS. Anti-oTP-1 antiserum was enriched for immunoglobins by precipitation with ammonium sulphate and subsequent fractionation on a DEAE-cellulose column (Good et al., 1980). Proteins were eluted with a 150 ml linear salt gradient (0-0.25 M NaCl in 10 mM Tris-HCl buffer, pH 8.2) and 2.0 ml fractions collected. The early eluting IgG-rich peak of anti-oTP-1 (fractions 3-19) was pooled and utilized in subsequent steps. Bovine CSP, purified oTP-1 or bovine serum albumin (50 μl of each per well) were allowed to adsorb passively to the wells of 96-well flexvinyl plates (Falcon brand; Fisher
Scientific, Orlando, FL) for 1 h at room temperature. These protein solutions were withdrawn, and plates washed once with 0.1% bovine serum albumin (w/v) in PBS (pH 7.4). Anti-oTP-1 antiserum or normal rabbit serum (NRS) then was added at increasing dilutions and allowed to stand in the wells at 4°C overnight. These solutions were removed and plates were washed with PBS containing bovine serum albumin. After washing, \(^{125}\text{I}\)-labelled, sheep anti-rabbit IgG antibody (50,000 c.p.m.; sp. act. 10^6 c.p.m./\mu g) which had been affinity purified was added to each well and left for 1 h at room temperature. The unbound labelled second antibody was then removed and the plates washed three times with PBS-bovine serum albumin. Wells were cut from the plates and the amount of bound \(^{125}\text{I}\) measured.

**Immunoprecipitation**

Duplicate aliquots of ovine and bovine conceptus culture medium (80 \(\mu\)g and 120 \(\mu\)g protein, respectively) were lyophilized and redissolved in immunoprecipitation buffer [0.35 ml 0.3 M NaCl, 0.05 M Tris-acetate (pH 7.5), 1 mM phenylmethylsulphonyl fluoride, 1 mM disodium EDTA, 0.1 mg bovine serum albumin/ml, 0.02% (w/v) Na\(_2\)N\(_3\) and 2% (v/v) Nonidet P-40]. Anti-oTP-1 antiserum (0.05 ml) or normal rabbit serum (0.05 ml) was added to ovine and bovine samples and the tubes were placed on a tube turner overnight at 4°C. Subsequently, 0.1 ml of a 10% (v/v) suspension of Protein A-Sepharose was added to each tube and allowed to incubate for 6 h at room temperature on a tube turner. The Sepharose suspension was centrifuged (15,000 g for 1 min) and washed five times with 1 ml detergent buffer [0.05 M Tris-acetate (pH 7.5), 0.5% (v/v) Nonidet P-40, 0.1% sodium dodecyl
sulphate, 0.3 M NaCl and 0.02% NaN₃]. Protein absorbed to the gel beads was solubilized in 0.05 ml 5 mM Tris-HCl (pH 6.8), 15% (w/v) glycerol, 5% (w/v) sodium dodecyl sulphate and 5% (w/v) 2-mercaptoethanol before one-dimensional electrophoresis, or in 0.05 ml 5 mM K₂CO₃, 5 mg dithiothreitol/ml, 2% (v/v) Nonidet P-40 and 9.16 M urea before two-dimensional electrophoresis. One-dimensional and two-dimensional polyacrylamide gel electrophoresis (1-D SDS-PAGE and 2-D SDS-PAGE), using 12.5% (w/v) acrylamide gels, were performed according to procedures described in detail by Roberts et al. (1984).

Isolation and Translation of Conceptus mRNA

Isolation and translation of conceptus mRNA was accomplished by the methods described by Hansen et al. (1985). Conceptus tissue was homogenized with 1 ml 4 M guanadinium thiocyanate, 0.5% (w/v) sodium N-lauroyl sarcosine, 25 mM sodium citrate buffer (pH 7.0), 0.1 M 2-mercaptoethanol and 0.1% (v/v) antifoam A, and then precipitated at -20°C with 25 µl 1 M acetic acid and 750 µl absolute ethanol (Chirgwin et al. 1979). The ethanol precipitate was collected by centrifugation (12,000 g), redissolved in the homogenization buffer (see above), layered over a 5.7 M cesium chloride cushion and centrifuged for 20 h at 100,000 g (20°C) to harvest total cellular RNA (Chirgwin et al., 1979). Polyadenylated RNA was isolated from the total cellular RNA by two cycles of binding to (50 mM potassium citrate, pH 7.5, 0.5 M potassium chloride, 1 mM disodium EDTA) and elution from 10 mM potassium citrate, pH 7.5, 1 mM disodium EDTA) oligodeoxythymidylate cellulose (Aviv & Leder, 1972).
Translation of conceptus poly A⁺ RNA was accomplished in a cell-free system using wheat-germ lysate (Roberts & Patterson, 1973). The translation mixture contained 0.25-1.0 μg poly A⁺ RNA, 6 μl wheat germ lysate, 48 mM potassium chloride, 27 mM Hepes (pH 7.5), 67 mM potassium acetate, 2.7 mM magnesium acetate, 1.2 mM ATP, 100μM GTP, 5.5 mM creatine phosphate, 200 μg phosphokinase/ml, 80 μM spermidine phosphate, 1 mM BME, 50 μM each of 19 amino acids (minus methionine), 600 μCi L-[³⁵S]methionine/ml and 13.5 μg placental RNase inhibitor/ml in a total volume of 15 μl. After translation, the [³⁵S]methionine-labelled products were analysed by 1-D SDS-PAGE as total translation products or following immunoprecipitation with anti-oTP-1 antiserum (10 μl) as described in the preceding section.

Results

Ouchterlony Double-Immunodiffusion Analysis

Rabbit antiserum prepared against highly purified oTP-1 gave a single immunoprecipitation band against both oCSP and bCSP (Fig. 2-la,b). When the ovine and bovine CSP were placed in adjacent outer wells of the immunodiffusion plate, with the antiserum in the central well, the immunoprecipitation band was discontinuous and had clearly defined spurs (Fig. 2-2).

Solid-Phase Radiobinding Assay

Total bCSP was adsorbed passively to the wells of flexvinyl plates. Anti-oTP-1 antiserum then was added at increasing dilutions and bound immunoglobulin detected by means of ¹²⁵I-labelled sheep antirabbit IgG (Fig. 2-3). The results confirmed that the anti-oTP-1
Fig. 2-1. Ouchterlony double-immunodiffusion analysis of conceptus secretory proteins from (a) sheep and (b) cattle. Total conceptus secretory proteins (a, sheep; b, cattle) (15 μl; 1.5 μg) were placed in the center well. Phosphate-buffered saline was placed in well 2 and antiserum to oTP-1 was placed in wells 3, 4, 5, 6 and 1 at increasing dilutions in phosphate-buffered saline (1:3, 1:7, 1:15, 1:31, 1:63) respectively.
Fig. 2-2. Ouchterlony double-immunodiffusion analysis of bovine and ovine conceptus secretory proteins. Anti-oTP-1 antiserum was placed in the center well (Godkin et al., 1982). Total bovine conceptus secretory proteins were placed in well 1 (30 μl, 6 μg) and wells 3 and 5 (15 μl, 1.5 μg). Total ovine conceptus secretory proteins were placed in wells 2, 4, and 6 (15 μl, 1.5 μg; 10 μl, 1.0 μg and 7.5 μl, 0.75 μg) respectively.
Fig. 2-3. Solid phase radiobinding assay of conceptus secretory proteins. Anti-oTP-1 antiserum was serially diluted and tested for binding to purified oTP-1 (-----), bCSPs (----), or bovine serum albumin (-----). Normal rabbit serum, used in a control, was serially diluted and tested for binding to purified oTP-1 (not shown), bCSPs (not shown) or bovine serum albumin ( --- -- ). Results of curves not shown were approximately 400 c.p.m. greater than the bovine serum albumin-non-immune rabbit serum at a 1:10 dilution and were similar at greater dilutions. Binding was measured using $^{125}$I-labelled sheep anti-rabbit IgG.
antiserum bound to some component in bovine CSP and crossreacted with purified oTP-1. At dilutions of antiserum below 1:20, there was detectable binding of antiserum to bovine serum albumin. Although the relative affinity of the antiserum for BSA was low compared to that of BCSP or oTP-1, the crossreactivity displayed here is biologically significant as BSA is a major component of conceptus cultures. Preimmune serum tested over a similar range of dilution failed to bind either oTP-1 or total bCSP. Half-maximal binding to bCSP and oTP-1 was detected at antiserum dilutions of about 1:80 and 1:160 respectively. The binding curves for these two protein fractions appeared parallel. At the initial 1:10 dilution of antiserum the wells containing adsorbed oTP-1 bound about twice as much 125I-labelled second antibody as did the wells containing bCSP.

Immunoprecipitation of Polypeptides from Ovine and Bovine CSP

Sheep and cattle conceptuses were incubated in the presence of L-[3H]luecine and polypeptides in the dialyzed culture medium immunoprecipitated by successive addition of anti-oTP-1 antiserum and protein A-Sepharose. For oCSP, the antiserum specifically crossreacted with polypeptide(s) with a M₆ about 19,000 (Fig. 2-4). With bCSP, two bands of polypeptides were detected by 1-D SDS-PAGE analysis. These bands had apparent molecular weights of 22,000 and 24,000 (Fig. 2-4). Gels were also stained with coomassie blue and after destaining specific precipitation of protein with relative molecular weight identical to BSA was visualized.

When the polypeptides from day 17-18 conceptuses (Fig. 2-5, upper panel) were immunoprecipitated and analysed by 2-D SDS-PAGE, 7
Fig. 2-4. Analysis of immunoprecipitates from conceptus secretory proteins of cows and sheep by one-dimensional polyacrylamide gel electrophoresis and fluorography. Lanes 1 and 3 contained material from bCSPs and oCSPs respectively, which had been immunoprecipitated by anti-oTP-1 antiserum followed by Protein A-Sepharose. Lanes 2 and 4 were control lanes in which bCSPs and oCSPs, respectively, had been treated with normal rabbit serum followed by Protein A-Sepharose.
Fig. 2-5. Analysis of bovine CSP by two-dimensional polyacrylamide gel electrophoresis and fluorography. Horizontal scale represents pH values. Top panel represents total array of proteins secreted by day 17-18 conceptuses. Lower panel represents immunoprecipitable material from bCSP. Bovine CSP was treated with anti-oTP-1 antiserum, immune complexes were collected on Protein A-Sepharose and the radioactive proteins analysed.
polypeptides could be visualized on fluorographs (Fig. 2-5, lower panel). The majority of these were localized in two parallel rows of apparent $M_\text{r}$ 22,000 and 24,000. Their approximate isoelectric points ranged from 6.7 to 6.5. These polypeptides were the major components present on gels of total bCSP at day 17-18 of pregnancy.

**Immunoprecipitation of Polypeptides from In-Vitro Translation Products**

Total poly (A)$^+$ conceptus mRNA was translated in vitro in a wheat-germ translation system in which L-[$^{35}$S]methionine was provided as a source of labelled amino acid. The products of translation were analysed by one-dimensional PAGE and fluorography. Translation of bovine mRNA gave rise to a range of translation products with molecular weights ranging from 130,000 to 12,500 (Fig. 2-6). During translation of ovine mRNA, the dominant translation product had a $M_\text{r}$ of about 21,000 (Hansen et al., 1985) and this component was specifically immunoprecipitated with anti-oTP-1 antiserum. For cow conceptus mRNA a dominant translation product of $M_\text{r}$ about 18,000 was noted which crossreacted with anti-oTP-1 antiserum.

**Discussion**

A considerable body of evidence has accumulated to suggest that the mechanisms involved in maternal recognition of pregnancy and luteal maintenance in the ewe and cow are similar (Roberts et al., 1985; Bazer et al., 1986; Thatcher et al., 1986a). Secretory components, proteinaceous in nature, are thought to be involved as antiluteolytic substances in both species. These substances probably act locally on the uterine endometrium and, by mechanisms still not
Fig. 2-6. Electrophoretic analysis of cell-free translation products of RNA isolated from cattle conceptuses. Lane 1 represents total translation products when no bovine mRNA was present. Lanes 2 and 3 show translation products when total poly (A)+ bovine conceptus mRNA was used as a source of exogenous mRNA. Lane 4 shows the material that was immunoprecipitated from such products by using normal rabbit serum. Lanes 5 and 6 show material that was immunoprecipitated from the total translation mixture by anti-oTP-1 antiserum. The proteins were analyzed in 12.5% polyacrylamide gels and detected by fluorography.
understood, reduce the pulsatile release of the presumed uterine luteolysin prostaglandin F-2, from the gravid uterine horn (Thatcher et al., 1986a; Fincher et al., 1985; Knickerbocker et al., 1986b). Other antiluteolytic, luteoprotective and luteotrophic mechanisms may be regulated by these unique proteins secreted by the conceptus (Thatcher et al., 1986b).

In both the ewe and cow, low molecular weight acidic proteins are major components of the conceptus secretions during the critical time that the corpus luteum is rescued. The question has arisen as to whether these proteins are antiluteolytic, either alone or in combination with other conceptus products. Until the present study these low molecular weight acidic polypeptides produced by the ewe and cow conceptus had not been compared.

Ouchterlony double immunodiffusion clearly showed that antiserum to oTP-1 crossreacted immunologically with some component(s) of bCSP. When ovine and bovine conceptus secretory proteins were placed in adjacent outer wells, with the antiserum to oTP-1 located in the center well, fusion of immunoprecipitation lines was incomplete and spurs were evident. This indicated that a protein is present in bCSP which is not identical to oTP-1, but is related serologically to it. This result was confirmed by solid-phase radiobinding assay which showed that half-maximal binding of antiserum to adsorbed oTP-1 occurred at about twice the antiserum dilution as observed with bCSP. The protein serologically related to oTP-1 in bCSP was clearly present as a high proportion of the total protein. In addition, binding curves obtained with increasing antiserum dilutions were
parallel, a result which suggests that relative affinity of antibody towards oTP-1 and crossreacting protein in bCSP is similar under these conditions. Recent evidence exists suggesting that the relative affinity of oTP-1 and the crossreactive component of bCSP are not similar. Vallet et al. (1988a) developed a radioimmunoassay for oTP-1 and was not able to detect displacement in the assay by undiluted bCSP while oTP-1 was detectable in oCSP diluted 1:1,000. Similarly, binding of bCSP to an immunoaffinity column constructed of IgG enriched, ant-oTP-1 antiserum, bound to cyanogen bromide activated sepharose, was very low and binding was inhibited by low salt concentrations (0.15 M) normally used to reduce non-specific binding. The fact that antiserum did bind to bovine serum albumin in the solid-phase radiobinding assay (Fig. 2-3) indicated that part of the reaction between bCSP and antiserum observed by ouchterlony immunodiffusion and solid phase binding assay may have been directed nonspecifically towards some plasma component. Crossreactivity against polypeptides released by day 12-14 pig conceptuses (Vallet et al., 1988a) has not been demonstrated. The antiserum does not, therefore, appear to have broad crossreactivity with trophoblast proteins from all species, but results should be viewed with caution due to its crossreactivity to BSA. Although anti-oTP-1 antiserum does not appear to be a probable aid in purification of crossreactive components of bCSP, it may still represent a useful tool for identification of crossreactive components of bCSP during characterization of these problems.
Anti-oTP-1 antiserum specifically immunoprecipitated a group of 6-8 polypeptides from bCSP which, when analysed by 2-D SDS-PAGE, fell into two major molecular weight classes (22 and 24 kDa). A trace of a 26 kDa component was also present. These bovine polypeptides were slightly more basic and of higher molecular weight than oTP-1 (Godkin et al., 1982). The fact that only a single molecular weight band (18,000 M) of protein is immunoprecipitated from cell-free translation of bovine poly (A)* mRNA by anti-oTP-1 antiserum suggests that heterogeneity in molecular size of the bovine protein may be the result of some post-translational processing of the initial translation product. This cell-free translation product has a relative molecular weight 4,000-6,000 less than the products immunoprecipitated from bCSP. This is in contrast to the immunoprecipitable translation product for ovine poly(A+) mRNA, in which the translation product has a molecular weight 4,000 larger than the products immunoprecipitated from oCSP (Hansen et al., 1985). These differences suggest that post-translation processing of the ovine and bovine proteins is dissimilar. One explanation is that bovine proteins are glycosylated whereas ovine protein simply undergoes proteolytic cleavage, eg. removal of signal sequence. In conclusion, results of this study demonstrate that the cow conceptus secretes a complex protein milieu part of which is immunologically related to oTP-1. Because of immunological similarity and because it is synthesized at a developmentally equivalent stage of pregnancy as oTP-1(Bartol et al., 1985), we suggest that the protein complex secreted by the bovine conceptus which crossreacts with antiserum to oTP-1 should be called bovine trophoblast protein-1 or (bTP-1).
CHAPTER 3
DIFFERENTIAL GLYOSYLATION OF THE COMPONENTS OF THE BOVINE TROPHOBLAST PROTEIN-1 COMPLEX

Introduction

The presence of a functional corpus luteum is essential for pregnancy maintenance in the cow and ewe. The conceptus, therefore, must signal its presence to attenuate uterine PGF-2α secretion that would otherwise lead to demise of the corpus luteum, termination of pregnancy, and resumption of ovarian cyclicity. For both the cow and ewe, signaling by the conceptus occurs via secretion of proteins (Godkin et al., 1984b; Knickerbocker et al., 1986b). The "signal" proteins are similar for both species since interspecies transfer of trophoblastic vesicles between the ewe and cow will cause cycle extension (Martal et al., 1984). In sheep, the anti-luteolytic molecule has been identified as oTP-1 (Godkin et al., 1984b). Cattle secrete immunologically cross-reactive molecules similar to oTP-1 called the bTP-1 complex. This complex consists of seven isomers secreted in two size classes (Chapter 2), appear to be members of the α-interferon family (Imakawa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988) and likely represent a novel role for interferon molecules.

While oTP-1 is not glycosylated (Anthony et al., 1988), bTP-1 appears to be glycosylated since conceptuses cultured with
[\(^{3}\text{H}\)]glucosamine incorporated the radioisotope into bTP-1 (Anthony et al., 1988). In addition, the primary translation product of bovine mRNA, which migrated as a single 17-18 kDa protein species during gel electrophoresis, was smaller than its secreted form (22 and 24 kDa; chapter 2; Anthony et al., 1988). Therefore, it seems likely that bTP-1 has undergone evolutionary divergence from the nonglycosylated ovine signal and other related interferons which are nonglycosylated, O-glycosylated and rarely N-glycosylated (Bielefeldt Ohmann et al., 1987; Langer and Pestka, 1985). Alternatively, bTP-1, oTP-1 and interferons may all represent molecules which arose from a common molecule and diverged simultaneously. The results of experiments, described in this chapter indicate that bTP-1 is glycosylated in an N-linked manner and that differences in relative molecular weight between classes of bTP-1 are due to differential post-translational processing to result in bTP-1 with high-mannose and complex-type carbohydrate moieties.

**Materials and Methods**

**Materials**

L-[\(^{35}\text{S}\)]methionine was from Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA); endo\(\beta\) -N-acetylglicosaminidase H (Endo H) from *Streptomyces plicatus* was purchased from Miles Laboratories (Naperville, IL); desoxymannojirimycin-HCl (DMM) from *Bacillus* species was obtained from Boehringer Mannheim (Indianapolis, IN); Conconavalin A-Sepharose 4B (Con A) and tunicamycin were from Sigma (St. Louis, MO), neuraminidase from *Vibrio cholerae* was from Life...
Technologies and endo α-N-acetylgalactosaminidase (O-glycanase) from *Diplococcus pneumoniae* was purchased from Genzyme (Boston, MA). All other materials were supplied as noted previously (chapter 2) or were reagent grade or better.

**In vitro culture of conceptuses**

Conceptuses collected from Angus and Brangus cows at day 17-18 of pregnancy were cultured as previously described (chapter 2) for 72 h with fresh Eagle's modified or complete minimum essential medium (MEM) being replaced every 24 h. Cultures were carried out with methionine-deficient (0.1x) medium supplemented with 50-100 μCi [35S]methionine or complete MEM with 100 μCi [3H] glucosamine/culture/24 h. Conceptus-conditioned medium was centrifuged for 15 min at 2,600 x g to remove particulate matter and dialyzed (M<sub>c</sub> cutoff = 3,500) extensively to remove low molecular weight compounds. Immunoprecipitation (IMP) of proteins in medium was carried out as previously described (chapter 2). One-dimensional and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D and 2-D SDS-PAGE), using 12.5% (w/v) polyacrylamide gels, were performed as described elsewhere (Roberts et al., 1984). Radioactive polypeptides were detected by fluorography using Kodak XAR film.

**Culture of conceptuses with tunicamycin**

Conceptuses were cultured in presence or absence of tunicamycin 20 μg/ml in MEM. Medium was prepared by adding 10 mg tunicamycin to 1 ml dimethylsulfoxide (DMSO) to create a 10 μg/μl stock, of which 100 μl was added to 50 ml methionine deficient MEM and filter sterilized.
After 24 h of culture, tissue and medium were separated by centrifugation and aliquants of medium dialyzed and lyophilized for analysis of total bCSP (50,000 cpm, 0.736 ml) and immunoprecipitable components of bCSP (200,000 cpm, 2.946 ml). Lyophilized total bCSP and immunoprecipitates were solubilized in gel loading buffer (chapter 2) in preparation for electrophoresis.

**Culture of conceptuses with DMM**

A stock solution of DMM (2.5 mg/ml) was prepared by dissolving 10 mg DMM in 4 ml methionine deficient MEM. Conceptuses were initially cultured in complete MEM for 24 h without treatment, and subsequently cultured for 4 h in the presence or absence of 1 mM (200μg/ml) DMM (1.2 ml of the 2.5 mg/ml stock solution added to 13.8 ml methionine deficient MEM). After 4 h of preincubation with DMM, 100 μCi L-[35S]methionine was added to culture medium. After 24 h of culture in presence or absence of DMM, tissue and medium were separated and medium dialyzed. Aliquants of dialyzed medium from control (total bCSP = 100,000 cpm, 0.561 ml; bCSP for immunoprecipitation = 350,000 cpm, 1.963 ml) and DMM-treated (total bCSP = 100,000 cpm, 0.242 ml; and bCSP for immunoprecipitation = 350,000 cpm, 0.847 ml) conceptuses were lyophilized in preparation for electrophoresis.

**Glycosidase treatment of conceptus culture medium**

Conceptus-conditioned medium from the second 24 h of culture in which conceptuses had been cultured in methionine deficient MEM supplemented with 100 μCi L-[35S]methionine were subjected to Endo H treatment. Endo H was prepared as a stock solution containing 6 μU/30 μl. Aliquants of total bCSP (50,000 cpm, 158 μl) and bCSP for
immunoprecipitation (200,000 cpm, 632 μl) were lyophilized. Samples were resolubilized with 0.47 ml of 0.1 M NaPO₄, pH 6.1, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 mM PMSF and 0.02% (w/v) NaN₃. Samples were incubated at 37 °C in presence or absence of Endo H (6 mU, 30 μl) for 24 h. At this time, Endo H (6 mU, 30 μl) was added again to appropriate tubes and incubation continued for an additional 24 h at 37°C. Enzyme digestions were stopped by boiling samples for 3 min. Total bCSP were then dialyzed and lyophilized in preparation for gel electrophoresis. Specified samples were immunoprecipitated as described in chapter 2 and then immunoprecipitates solubilized with gel loading buffer for electrophoretic analysis.

In a separate experiment, conceptus-conditioned medium from the second 24 h of culture was utilized for incubation with neuraminidase with or without O-glycanase. Neuraminidase was obtained containing 500 U/ml. The stock solution was diluted (2 μl in 18 μl H₂O) to obtain a working solution containing 0.1 U/μl. O-glycanase was purchased as a 1,450 mU/ml solution and 2 μl (2.8 mU) used in incubations. Aliquots of total bCSP for 1-D SDS-PAGE (100,000 cpm; 73 μl) and 2-D SDS-PAGE (200,000 cpm; 145 μl) and of bCSP for subsequent immunoprecipitation and 1-D SDS-PAGE (300,000 cpm; 218 μl) and 2-D SDS-PAGE (600,000 cpm; 435 μl) were lyophilized. Fetuin (an O-linked glycoprotein) and asialofetuin (a deglycosylated form of fetuin) were used as controls for confirmation of enzyme activity (20 g/10 μl in 20 mM Tris-maleate, pH 6.0). Samples were then resolubilized with 46 μl 20 mM Tris-maleate, pH 6.0, 10 mM D-
galactono-1,4-Lactone, 1 mM calcium acetate. Neuraminidase (0.1 U) was added to appropriate tubes and incubated at 37°C for 1 h and then 0-glyconase (2.8 mU) added and incubation continued at 37°C overnight. Reactions were stopped by adding 50 μl gel loading buffer to samples which were then separated by gel electrophoresis.

**Concanavalin A-Sepharose 4 B lectin chromatography**

A 2 ml Con A lectin column was poured (14 mg Con A/ml packed gel) and equilibrated with wash buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂ and 0.02% (w/v) NaN₃). Conceptus-conditioned medium from the second 24 h of culture in which conceptuses were cultured in methionine-deficient MEM supplemented with 100 μCi L-[³⁵S]methionine was utilized. Conceptus-conditioned medium which had been dialyzed against distilled H₂O (8.1 x 10⁵ cpm, 8.426 ml) was diluted 1:2 with wash buffer, passed over the column and washed with 20 column volumes of buffer. Material from the column was then eluted with 0.1 M α-methyl-D-glucoside (in wash buffer) followed by 0.1 M α-D-methyl-mannoside (in wash buffer) and finally with 6 guanidine-HCl, pH 3.1. Unfractionated bCSP, material not binding to the matrix and eluted material were dialyzed and lyophilized in preparation for separation by 1-D SDS-PAGE.

**Results**

**Glycosylation of bTP-1**

Culture with [³H] glucosamine (Fig. 3-1) demonstrated that proteins migrating in the region of bTP-1 were glycosylated (Total lane). Immunoprecipitation of bTP-1 with antiserum to oTP-1 resulted
Fig. 3-1. Fluorograph of SDS-PAGE of conceptus supernatants from a conceptus cultured in medium containing 100μCi [³H] glucosamine. After 24 h of culture, tissue and medium were separated and medium was dialysed extensively and lyophilized. The first lane of the figure represents the total array of proteins present in the culture supernatants. Aliquants of dialysed medium were immunoprecipitated with antiserum to oTP-1 or normal rabbit serum as indicated in the figure (Chapter 2). Samples were separated by SDS-PAGE in presence of 2-mercaptoethanol.
in visualization of one immunoreactive species migrating at 24 kDa. The lower molecular weight form of bTP-1 was not visualized utilizing this method.

**Inhibition of Glycosylation during In Vitro Culture of Conceptuses**

A bovine conceptus was incubated with L-[^35S]methionine in the presence of tunicamycin (20 μg/ml) to inhibit N-linked glycosylation. Without tunicamycin, two molecular weight classes of bTP-1 could be immunoprecipitated from conditioned culture medium (22 and 24 kDa). In the presence of tunicamycin, the conceptus secreted only one labelled species (18 kDa) that could be immunoprecipitated from culture medium (Fig. 3-2, lane 5). A conceptus also was cultured in the presence of DMM (1 mM) for 24 h to specifically inhibit N-linked, complex, glycoprotein formation. Only one molecular weight species of bTP-1 (22 kDa) was detected in culture supernatants from the DMM-treated conceptus (Fig. 3-3). The 24 kDa form of bTP-1 was absent.

**Glycosidase Treatment of Conceptus Proteins**

Conceptus culture supernatants were incubated for 48 h with Endo H, which cleaves the two N-acetylgalactosamine residues of the chitobiose core of high mannose, N-linked glycoproteins (Tarentino et al., 1974). Treatment with Endo H resulted in a shift in molecular weight of the 22 kDa form of bTP-1 to approximately 20 kDa (Fig. 3-4), but had no affect on the electrophoretic mobility of the 24 kDa form of bTP-1. In a parallel experiment, conceptus culture supernatant from the second 24 h of culture was incubated in the presence of neuraminidase (0.1 U/50 μl) with or without O-glycanase
Fig. 3-2. Fluorograph of SDS-PAGE of conceptus supernatants from conceptuses cultured in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of tunicamycin for 24 h. Conceptuses were cultured in methionine-deficient medium containing 100 μCi L-[^35S] methionine. Aliquots of dialyzed medium were immunoprecipitated with antiserum to oTP-1 (lanes 3 and 5) or normal rabbit serum (lanes 4 and 6) as previously described (chapter 2) and separated by SDS-PAGE in the presence of 2-mercaptoethanol. Fluorographs were exposed to the dried gel for various times to maximize detection of individual proteins present.
Fig. 3-3. Fluorograph of SDS-PAGE of conceptus supernatants from conceptuses cultured in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of DMM for 24 h. Aliquots of medium were immunoprecipitated with either antiserum to oTP-1 (lanes 3 and 5) or normal rabbit serum (lanes 4 and 6) as previously described (Chapter 2) and separated by SDS-PAGE in the presence of 2-mercaptoethanol.
Fig. 3-4. Fluorograph of SDS-PAGE of conceptus proteins treated with Endo H. Samples were incubated at 37°C in the presence (lanes 2, 5, 6) or absence (lanes 1, 3, 4) of Endo H. Samples were then immunoprecipitated using either antiserum to oTP-1 (lanes 3 and 5) or normal rabbit serum (lanes 4 and 6) and resolved on 12.5% (w/v) polyacrylamide gels in the presence of 2-mercaptoethanol. Fluorographs were exposed to the dried gel for various times to maximize detection of individual proteins present.
2.8 mU/50 μl) to assess whether bTP-1 contained sialic acid residues or O-linked carbohydrate chains (Lamblin et al., 1984). Neither enzyme treatments altered the molecular weights or isoelectric points of proteins in the bTP-1 complex as discerned by electrophoretic migration following 1-D or 2-D SDS-PAGE (data not shown).

Concanavalin A-Sepharose Column Chromatography

Conceptus-conditioned culture medium was passed over a 2 ml Con A column. Although the bulk of the 24 kDa form of bTP-1 passed freely through the column matrix, none of the 22 kDa form did so (Fig. 3-5). No appreciable amount of radioactive material was removed from Con A by elution with 0.1 M α-methyl-D-glucoside or 0.1 M α-D-methyl-mannoside (data not shown). Elution with 6 M guanidine-HCl, pH 3.1, resulted in elution of all of the 22 kDa form of bTP-1. A small proportion of the 24 kDa form of bTP-1 was released from Con A; this represented a very small fraction of the 24 kDa form found in crude culture medium or when compared to material not binding to Con A (Fig. 3-5).

Discussion

It has been shown that a component of conceptus secretory proteins is responsible for extension of luteal lifespan in the cow and ewe (Godkin et al., 1984b; Knickerbocker et al. 1986b). Extension of luteal lifespan in the ewe is due to secretion of oTP-1 by the conceptus (Godkin et al., 1984b; Vallet et al., 1988b), a molecule which is immunologically related to the bovine conceptus complex called bTP-1 (chapter 2).
Fig. 3-5. Concanavalin A-Sepharose 4B lectin chromatography of bTP-1. Samples of protein from crude conceptus-culture supernatant, material not binding to the column and eluted material were immunoprecipitated with antiserum to oTP-1 (lanes 1, 2, and 3, respectively) and immunoprecipitates resolved by SDS-PAGE and fluorography using 12.5% (w/v) polyacrylamide gels in the presence of 2-mercaptoethanol. No proteins were visualized when samples were immunoprecipitated with normal rabbit serum.
These immunologically-related proteins differ in size (oTP-1, 19 kDa; bTP-1, 22 and 24 kDa) and isoelectric variants [oTP-1, pI=5.4-5.7; bTP-1, pI=6.5-6.7, (chapter 2)]. Whereas, oTP-1 is not glycosylated (Anthony et al., 1988), bTP-1 is, glycosylated.

Culture of bovine conceptuses with tunicamycin, an inhibitor of N-linked glycosylation (Tkacz and Lampen, 1975), resulted in loss of immunoprecipitable bTP-1 in the 22-24 kDa range with concomitant appearance of immunoreactive molecules migrating at 18 kDa. These results indicate that both forms of bTP-1 are N-linked. These data are also consistent with molecular weight estimates of bTP-1 produced from translation of bovine conceptus mRNA by wheat germ lysate [17-18 kDa; (chapter 2; Anthony et al., 1988)]. Conceptus secretory proteins were incubated with neuraminidase, since removal of terminal sialic acid residues is required for O-glycanase to be effective, and then incubated with O-glycanase, an enzyme which cleaves carbohydrates that are linked to the polypeptide chain via serine or threonine (Lamblin et al., 1984). Neuraminidase and O-glycanase were effective in removal of sialic acid residues and cleavage of O-linked chains, respectively, from control glycoproteins (fetuin, asialofetuin; data not shown), but enzyme treatments had no effect on bTP-1. Therefore, bTP-1 does not contain O-linked carbohydrate moieties or detectable quantities of terminal sialic acid residues.

Several lines of evidence indicate that the two molecular weight classes of bTP-1 arise from post-translational processing of a common transcript (18 kDa) to form a 22 kDa high-mannose form and a 24 kDa complex-type form. First, culture of conceptuses in the presence of
DMM, an inhibitor of mannosidase I (Fuhrmann et al., 1984), which processes high-mannose forms to complex-type oligosaccharides, blocked synthesis of the 24 kDa form, but not the 22 kDa form of bTP-1. Second, treatment of conceptus secretory proteins with Endo H resulted in reduction of the 22 kDa form of bTP-1 to 20 kDa but did not affect the 24 kDa form. Finally, experiments utilizing Con A to bind high-mannose units (Cummings and Kornfeld, 1982), indicated that the majority of the 24 kDa form of bTP-1 did not bind to Con A. Conversely, all of the 22 kDa form of bTP-1 was bound to Con A and could only be removed by elution with 6 M guanidine-HCl. A small proportion of the 24 kDa form did bind to Con A, perhaps because of some non-specific binding or because a subpopulation of the 24 kDa form may contain sufficient numbers of mannose moieties to allow some binding to Con A. Difficulty was encountered in obtaining sufficient guanidine eluted material for analysis by electrophoresis. When guanidine eluted material was placed in dialysis at 4°C, precipitate (most likely guanidine-HCl) appeared in the dialysis tubing and would not completely dissolve during dialysis. The majority of radioactivity was trapped in the precipitate and was not used for analysis. Therefore, some caution is required during analysis of electrophoretic gels because it is not known which proteins were trapped, which might bias interpretations of the results.

Glycosylation of bTP-1 is unique for several reasons. The related molecule, oTP-1, is not glycosylated. Secondly, oTP-1 and probably bTP-1 are members of the α-interferon family (Imakawa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988), and few α-interferons
are glycosylated. Pestka reported that all but one species of human α-interferon lack the Asn-x-Ser/Thr sequence necessary for N-glycosylation and that O-glycosylation is more common for α-interferons (Pestka et al., 1987). Only one site exists on oTP-1 for N-glycosylation (Imakawa et al., 1987). The glycosylation of bTP-1 may reflect simple evolutionary divergence. The role of glycosylation may be important for increasing the half-life and stability of bTP-1 once secreted by the conceptus into the uterine lumen, or could affect binding of bTP-1 to its endometrial receptor or initiation of its biological effects. Finally, glycosylation of bTP-1 is of interest because the differential processing documented here appears to account for the molecular weight variants in the bTP-1 complex of proteins. It remains to be determined whether isoelectric and molecular weight variants have differing biological properties. Further studies on characteristics and biological (antiluteolytic) effects of bTP-1 require an effective means for purification of this putative conceptus signal.
CHAPTER 4
INTRAUTERINE INFUSION OF PURIFIED BOVINE TROPHOBLAST PROTEIN-1 COMPLEX EXERTS AN ANTILUTEOLYTIC EFFECT AND EXTENDS CORPUS LUTEUM LIFESPAN IN CYCLIC CATTLE

Introduction

Continued maintenance of the CL is required if maintenance of early pregnancy is to occur in cattle. Failure of a conceptus to "signal" its presence results in PGF-2 release, luteolysis, and resumption of ovarian cyclicity. The bovine conceptus must signal its presence by days 15 to 17 of pregnancy if CL function is to be maintained (Northey & French, 1980; Betteridge et al., 1984; Humblot & Dalla Porta, 1984). Attenuation of PGF release and extension of CL lifespan are accomplished by secretion of bCSP (Knickerbocker et al., 1986a,b). Therefore, bTP-1, which represents a major component of bCSPs from day 17-18 conceptuses (Bartol et al., 1985; chapter 2), has been identified as a candidate for the antiluteolytic agent in cattle. Also, it is immunologically related to oTP-1 (chapter 2), which is responsible for luteal maintenance in the ewe (Godkin et al., 1984b; Vallet et al., 1988b). This bTP-1 complex of proteins consists of seven isoelectric variants (pI = 5.5 to 5.7) of N-linked glycoproteins, in two molecular weight classes; a 22 kDa high-mannose type and a 24 kDa complex-carbohydrate type (chapter 2; Anthony et al., 1988). The present study was designed to evaluate the role of the bTP-1 complex in extension of CL lifespan. Objectives were to
purify bTP-1 and test its efficacy as an antiluteolysin when infused into the uterine lumen during days 15.5 to 21 of the estrous cycle.

Materials and Methods

Materials

Tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, New York. Eagle's minimum essential medium (MEM, Gibco custom formula #86-5007) for culture of conceptuses was prepared according to procedures described in chapter 2. Acrylamide, dithiothreitol and sodium dodecyl sulfate were obtained from Research Organics, Inc., Cleveland, Ohio; L-[4,5-3H] leucine was from Amersham, Arlington Heights, IL; N,N'- diallyltartardiamide and ammonium persulfate were from Bio Rad Laboratories, Richmond, CA; Coomassie Blue R-250 was purchased from Polysciences, Inc., Warrington, PA; urea (ultrapure) was obtained from Schwarz/Mann, Cambridge, MA; ammonium sulfate was obtained from Serva, Heidelberg, N.Y.; Aquacide II was from Calbiochem, San Diego, CA; X-OMAT AR film was from Eastman-Kodak, Rochester, N.Y.; N,N,N',N'-tetramethylenediamine, pepstatin-A, leupeptin, phenylmethylsulfonyl fluoride (PMSF), Nonidet P-40, ovalbumin, and myoglobin were purchased from Sigma Chemical Co., St Louis, MO; bovine albumin, Fraction V was from United States Biochemical Corp., Cleveland, Ohio; carboxymethyl (CM)-Sepharose and DEAE Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; bicinchoninic acid was from Pierce, Rockford, IL; 2-mercaptoethanol was from Fisher Scientific, Orlando, FL; and ampholytes were obtained
from Serva, Bio Rad or LKB. All other chemicals were reagent grade or better.

**In-Vitro Culture of Conceptuses**

Conceptuses were collected from Angus and Brangus beef cows at day 17-18 after estrus (estrus = day 0) and were cultured as described previously (chapter 2) for 72 to 96 h, with medium being replaced every 24 h. Cultures were carried out with either complete MEM, leucine-deficient (0.1 x) MEM supplemented with 100 μCi [³H] leucine/culture/24 h, or in methionine-deficient (0.1 x) MEM supplemented with 100 μCi [³⁵S] methionine/culture /24 h (chapter 2). Culture supernatants were frozen at -20°C until utilized.

**Purification of bTP-1 Complex**

Sat**ed ammonium sulfate precipitation of conceptus-conditioned culture medium.** A total of 95 conceptus culture equivalents (the material harvested from a conceptus incubation during 24 h) from the first (n=36), second (n=34) and third (n=25) 24 h of culture were collected. Conceptus-conditioned culture medium was pooled (approximately 1,350 ml; 14.2 ml per culture equivalent) and centrifuged for 15 min at 2,600 x g to remove particulate matter. From this pool, fluid representing 22 culture equivalents was set aside for infusion into cows receiving bCSP treatment. Thirty milliliters of bCSP from conceptuses cultured in the presence of [³H]-leucine (21 x 10⁶ dpm radioactivity) were added to the remaining 73 culture equivalents (approximately 1,068 ml total volume) to monitor subsequent yields during purification. An equal volume of saturated ammonium sulfate (SAS) was added to the medium, incubated
for 2 h at 4°C, and centrifuged (13,000 x g) for 30 min. After harvesting the supernatant, precipitates were resuspended in a total volume of 20 ml 20 mM Tris-HCl, pH 8.2 and the SAS precipitation procedure repeated. Supernatants from the second procedure were pooled with supernatants from the original precipitation. This material was buffered with 10 mM acetate, pH 5.0 containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.7 mg/l pepstatin-A, 0.5 mg/l leupeptin and 0.02% (w/v) NaN₃ and dialysed (M<sub>r</sub> cutoff = 6,000-8,000) against eight changes of 6 l volumes of 10 mM acetate buffer (pH 5.0) containing 1 mM EDTA, 0.2 mM PMSF, 0.7 mg/l pepstatin-A and 0.02% NaN₃. Supernatants were dialyzed further against two changes of 6 l volumes of 20 mM Tris-HCl, pH 8.2 with 1 mM EDTA, 0.7 mg/l pepstatin-A, 0.5 mg/l leupeptin and 0.02% NaN₃. After dialysis, the material was adjusted to pH 8.2 by addition of 1 M NaOH in preparation for CM-Sepharose column chromatography. Because of the influx of water due to osmotic pressure differences in initial dialysis steps, volume of the material was approximately doubled during the dialysis process.

**Ion exchange chromatography.** All buffers used for ion exchange chromatography contained a cocktail of protease and bacterial growth inhibitors composed of 1 mM EDTA, 0.7 mg/l pepstatin-A, 0.5 mg/l leupeptin, and 0.02% (w/v) NaN₃. Supernatants from SAS precipitation were passed over a 2.6 cm x 3.6 cm CM-Sepharose column equilibrated in 20 mM Tris-HCl, pH 8.2. Bound proteins were eluted subsequently using 20 mM Tris-HCl pH 8.2 containing 1 M NaCl. Material not binding to the column was further processed by anion exchange
chromatography. Two DEAE columns (2.6 cm x 3.6 cm) equilibrated with 10 mm Tris-HCl, pH 8.2, were attached in series so that if column capacity of the first column was exceeded, proteins running through would be bound on the second column. Material not binding to the CM-Sepharose column was then passed over the series of DEAE columns. Each column was washed extensively with 20 mM Tris-HCl, pH 8.2 to remove proteins not bound to the column. Bound proteins were eluted from each of the two DEAE columns separately, using 0.2 M sodium phosphate buffer, pH 7.9. First, one column volume of buffer was run through each column and flow stopped for a period of 2 h. Column flow was then continued and fractions pooled based upon presence of radioactivity. Pooled fractions were concentrated from 161 ml to 22.7 ml in a period of 24 h by placing the sample in dialysis tubing (Mr cutoff = 3500) and packing the tubing in Aquacide II (carboxymethyl cellulose) to remove the buffer. Samples (1 ml aliquants) were stored at -70°C until separation by HPLC gel filtration.

High pressure liquid chromatography. In order to minimize ionic interaction with the Zorbax GF-250 gel filtration column, samples were always prepared with sodium phosphate at a concentration of at least 0.2 M. This buffer was designed to reduce non-specific ionic interactions with hydroxyl groups on the silica based HPLC column, while utilizing a biologically compatible buffer to maintain stability of the column packing material and preserve sample integrity. To assure concentrations of this magnitude, 0.1 M sodium phosphate (monobasic=0.082 g/100ml and dibasic=1.370 g/100ml) was
added to concentrated samples and pH was adjusted to 7.9. Sodium phosphate was added because samples eluted from the DEAE column were contained in a mixture of sodium phosphate buffer and DEAE column buffer which resulted in a concentration of sodium phosphate less than 0.2 M. Separation was achieved using a system consisting of a Series 4 Liquid Chromatograph Microprocessor Controlled Solvent Delivery System (Perkin-Elmer; Norwalk, CT), a Series 4 Control Module to control the delivery system (Perkin-Elmer), an ISS-100 Intelligent Sampling System with refrigerated sample tray (Perkin-Elmer), a Zorbax GF-250 gel filtration column (DuPont Co.; Wilmington, DE), a LKB 2140 Rapid Spectral Detector (LKB; Bromma, Sweden), and a LKB 2211 SupeRac fraction collecting system, custom modified to include a refrigerated collection tray. Elution profiles of proteins were analyzed on an IBM XT computer connected to the spectral detector. Mobile phase of the column was 0.2 M sodium phosphate buffer, pH 7.9 with a flow rate of 0.7 ml/min. Column pressure ranged from 5 to 15 mega pascal (1 mega pascal = 150 psi). Aliquants of sample (175 µl) were passed over the HPLC gel filtration column and 4 drop fractions (230 µl) collected. Injections completed during the course of one day were collected into one set of fraction receptacles (5 ml) maintained at 4°C. At the end of each daily collection period, fractions were frozen. Once all of the sample had been processed in this fashion, fractions corresponding to elution volumes of protein having molecular weights of approximately 20 to 29 kDa were pooled.
Other Procedures

Preparation of radiolabelled conceptus-conditioned culture medium for direct analysis. Culture medium from conceptuses incubated with \(^{3}\text{H}\)leucine or \(^{35}\text{S}\)methionine were centrifuged (2,600 x g) for 15 min to remove particulate matter and then dialyzed extensively against 4 changes of 4 l of distilled water in dialysis tubing (Mr cutoff = 3,500) to remove low molecular weight compounds. Samples were then lyophilized as needed.

Polyacrylamide gel electrophoresis. One-dimensional and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D and 2-D SDS-PAGE) were performed utilizing the buffer system of Laemmli (1970) using 12.5 % (w/v) polyacrylamide gels. Samples were reduced with 5 % (v/v) 2-mercaptoethanol before electrophoresis. Radioactive polypeptides were detected in gels impregnated with 1 M sodium salicylate using Kodak XAR film by fluorography (Roberts et al., 1984).

Protein determination. Protein concentrations were determined by the BCA protein assay using BSA as the standard (Smith et al., 1985).

Preparation of materials for intrauterine infusion. All materials were prepared in 0.2 M NaPO\(_4\) buffer, pH 7.4 with penicillin and streptomycin (P/S; 100 U/ml and 100 g/ml) added. Bovine serum albumin was prepared at a concentration of 1.5 mg per 2.134 ml for infusion. Bovine conceptus proteins (n=22 culture equivalents) were dialyzed extensively against 10 mM NaPO\(_4\), pH 7.4 containing P/S and then concentrated as described previously using Aquacide II. Sodium phosphate (0.2 M), BSA and P/S were added to the concentrate and pH
adjusted to 7.4. Infusion doses contained 0.75 mg bCSP and 0.75 mg BSA per 2.134 ml. Purified bTP-1 was prepared by aliquanting 35 μg bTP-1 and 1.465 mg BSA per 2.134 ml. All aliquants were frozen at -70°C until time of intrauterine infusion.

Confirmation of purity. Aliquants of bovine serum albumin (BSA; 20 μg), bCSP (100 μg) and purified bTP-1 (16 μg) were concentrated and desalted using AMICON 10 ultrafiltration devices (AMICON, Danvers, MA). Samples were resolubilized with 0.05 ml 5 mM K₂CO₃, 5 mg dithiothreitol/ml, 2% (v/v) Nonidet P-40 and 9.16 M urea and analyzed by two-dimensional SDS-PAGE. Gels were equilibrated in 50% methanol and protein bands identified by silver staining procedures described by Wray et al. (1981). Immunoblotting was used to identify immunoreactive bTP-1 in the purified array of proteins. Purified bTP-1 complex was separated by 2-D SDS-PAGE (8 μg/gel) and transferred to nitrocellulose (0.2 μm; Scheicher & Schull). Blots were stained for 5 min to identify protein standards using 0.05% (w/v) amido black in methanol:acetic acid:water (40:10:50; v/v/v) and destained for 2 min in the same solvent. Blots then were washed briefly with water and placed in blocking buffer (10 mM Tris-HCl, pH 7.4 containing 2% (w/v) gelatin, 0.15 M NaCl, 0.01% (v/v) Tween 20 and 0.02% (w/v) sodium azide) for 8 h. Blots were subsequently incubated with either rabbit antiserum to anti-oTP-1 (diluted 1:100) or normal rabbit serum (NRS; diluted 1:100) for 2 h in incubation buffer (10 mM Tris-HCl, pH 7.4 containing 1% (w/v) gelatin, 0.15 M NaCl, 0.01% (v/v) Tween 20 and 0.02% sodium azide). Blots were rinsed with water, washed in incubation buffer twice for 30 min and
then incubated with 20 ml of $^{125}$I-Protein A ($1 \times 10^6$ cpm/ml; prepared as described by Hansen and Newton, 1988) in incubation buffer for 1 h, washed extensively with incubation buffer and allowed to dry. Autoradiography was performed using Kodak XAR film and enhancing screen for visualization of immunoreactive protein bands.

**Animal preparations.** Cyclic, non-lactating Jersey cows ($n=9$) were assigned randomly to receive intrauterine infusion of either BSA (control), bCSP or bTP-1 treatment. They were prepared for surgical placement of uterine catheters by a modification of procedures described by Knickerbocker et al. (1986b) on day 11 ($n=3$) or day 12 ($n=6$) of the estrous cycle. Treatments were randomized within day of estrous cycle on which surgery was performed. Utilizing midventral laparotomy, the uterus and ovaries were exposed and location of the CL recorded. A sterile polyvinyl catheter (V-6; Bolab Incorporated, Lake Havasu City, AZ) was inserted and secured approximately 2 cm into the anterior lumen of the uterine horn ipsilateral to the CL and exteriorized via a small flank incision. A small piece of surgical tape was wrapped around each catheter at the point of exit from the body cavity and anchored to the skin with a suture. The uterine catheters were placed in a pack, consisting of a 10 x 16 cm ziplock bag reinforced with surgical tape, and secured to the flank with sutures. Antibiotic (Tylan, 200 mg/ml; Elanco Products Co., Indianapolis, IN) was administered on the day of surgery (10 ml; i.m.).

Intrauterine infusions were administered at 12 h intervals ($1.5$ mg protein per infusion) from 1900 h on day 15 to 0700 h on day 21.
Blood was collected via jugular venipuncture every other day from day 7 to 13. On day 15, all cows were fitted with an indwelling jugular catheter. A jugular vein was punctured with a 12 guage needle and a sterile polyvinyl catheter (V-9; Bolab Incorporated, Lake Havasu City, AZ) threaded through the needle barrel into the jugular vein. The needle was then withdrawn leaving approximately 46 cm of catheter in the jugular vein. The catheter was flushed with sterile, heparinized saline (200 units/ml) to monitor catheter patency. The catheter was then threaded through a 10 cm² piece of foam rubber with a hole in its center which was attached to the neck with adhesive (KaMar, Steamboat Springs, CO). A section of catheter remaining outside the neck (30 cm) was secured between layers of Elastikon tape (Johnson & Johnson, Inc; New Brunswick, NJ). Remaining catheter (46 cm) was coiled in a plastic pouch sealed between the Elastikon layers. Blood samples were collected into heparinized tubes (100 units) twice daily (0730 and 1930 h) from day 15 until 24 h after onset of estrus and plasma harvested (20 min. 3,000 rpm, 4°C).

Catheters also were placed into the posterior vena cava immediately anterior to the uterine drainage on day 18 of the estrous cycle by a modification of the procedure described by Sears et al. (1978). Polyethylene catheters (120 cm; i.d. 0.86 mm, o.d. 1.27 mm) were inserted 70 cm into the tail vein via a 14 gauge disposable needle and secured to the tail of each animal with Elastikon adhesive tape. The remaining 50 cm of catheters was inserted into the palm of a vinyl glove from which the fingers had been removed and the glove taped in place. During blood sampling, the cannulae were removed from
the wrappings and secured to the tail head with Elastikon tape. Blood samples (10 ml) were taken every 15 min from 0900 h to 1500 h on days 19, 20 and 21 of the estrous cycle. Sample collections were discontinued prior to day 21 if a cow was observed in estrus. The day of estrus represented the last day of sampling. Samples were placed in an ice bath, centrifuged (20 min, 3,000 rpm, 4°C) within 15 min of collection, and plasma frozen.

Animals were maintained in a concrete pen from day 7 of the estrous cycle until surgery was performed. Animals were then housed in stanchions for duration of the experiment. Paint was applied to the tail head of all cows and crayon applied over this to facilitate estrus detection (Macmillan et al., 1988). Cows were observed for estrus in a dirt lot twice daily (0800 and 2000 h) for 30 min from day 15 until 24 h after the last cow was detected in estrus. At this time, all intrauterine catheters were cleaned, withdrawn from the body cavity as far as possible by gentle traction, cut and allowed to pass into the body cavity. Animals were then maintained on pasture until observed in estrus.

Radioimmunoassay of plasma hormones. Progesterone concentrations in heparinized plasma samples were measured using the assay system described by Knickerbocker et al. (1986b). Utilizing an antiserum dilution of 1:40,000, sensitivity of the assay was 31.2 pg per tube. Assays were carried out utilizing 100 or 200 μl of sample. Intra- and interassay coefficients of variation were 7.11% and 13.41%, respectively.
Heparinized plasma samples from the posterior vena cava were assayed for PGF by procedures described by Knickerbocker et al. (1986c) for unextracted samples. The assay was modified to use an antibody supplied and characterized by Kennedy et al. (1985); crossreactivities of the PGF antiserum were described previously by Gross et al. (1988). Sensitivity of the assay procedure was 10 pg/ml with an antibody dilution of 1:5,000. Unextracted plasma samples were assayed for PGF using aliquants of 200 µl. Accuracy of the procedure for unextracted plasma was characterized by measuring known quantities of exogenous PGF added to 200 µl of plasma (from cattle treated with an inhibitor to cyclooxygenase; Guilbault et al., 1984) at concentrations of 10-5,000 pg per tube [Y= 18.0 + 1.06x ; Y=amount of PGF measured (pg/0.2 ml), and x=amount of PGF added (pg/0.2 ml), R²=0.98]. A quantitative linear displacement curve was achieved. Test for homogeneity of regression between this curve and the standard curve indicated that the curves were parallel. The intra- and interassay coefficients of variation were 10.6% and 18.5%, respectively.

Statistical analysis. Data for progesterone concentrations in plasma were analysed using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Incorporated, 1985) for a split-plot analysis of variance with repeated measurements over time. The analysis of variance considered variability due to treatment (bTP-1, bCSP and BSA control), cow nested within treatment, sample time, treatment by time interaction and residual. To provide further estimates of temporal changes, progesterone concentrations were
analysed by least squares regression analyses, and differences in treatment means and regression curves were evaluated by orthogonal contrasts (BSA vs bCSP and bTP-1; bCSP vs bTP-1). For example, differences in polynomial regression curves were tested by examining for homogeneity of regression between treatment response curves. These data were analysed with time as a continuous, independent variable. Data pertaining to interestrous interval during the treatment estrous cycle, period from pre-treatment estrus until when progesterone fell below 1 ng/ml, period from when progesterone fell below 1 ng/ml to subsequent estrus, and post-treatment interestrous interval were analysed by least squares analysis of variance. Differences between treatment means were evaluated by orthogonal contrasts described above.

Data pertaining to plasma PGF concentrations were evaluated by analysis of variance with treatment, cow nested within treatment, and residual considered in the model. Day was not considered due to the unbalanced distribution of cows among days. For day 19, 2 BSA, 2 bCSP, and 2 bTP-1 cows were represented. For cows on day 20, 1 BSA and both bTP-1 cows were represented, and on day 21, only 2 bTP-1 cows were represented. Analysis of the data indicated that variances among treatment groups were heterogeneous. Data were then transformed by taking the reciprocal of PGF concentration and differences for treatment means examined; variability due to treatment and cow nested within treatment were examined. Other transformations of data were examined (Log, square root), but
reciprocal of PGF concentrations reduced the heterogeneity of variance the most.

Results

Characteristics of the Culture System

Conceptus culture supernatants from the first, second, third, and fourth 24 h culture periods (n = 3), in which MEM and leucine were replaced every 24 h, were analysed for protein concentration and incorporation of radiolabel into nondializable molecules (Table 4-1). Incorporation rate of leucine into nondialyzable secreted proteins and protein content of culture supernatants tended to increase from the first to second 24 h culture period, remained constant through the third day of culture and declined the fourth day of culture. The observed increase in percent incorporation from the first to second and third culture periods was likely the result of dilution of the leucine pool of tissue during the first 24 h of culture. As labelled leucine was carried into the tissue and utilized, increased incorporation rates would be observed later in culture. Samples from one conceptus were analysed to evaluate qualitative patterns of radiolabelled macromolecules as determined by HPLC gel filtration chromatography (Fig. 4-1). In order to normalize data to account for disproportionate recovery of material for each culture period (61.02%, 55.13%, 45.02%, and 68.12% recovery for first, second, third, and fourth 24 h culture supernatants, respectively), data were represented as percent of total radioactivity recovered from the amount injected onto the column (25,000 dpm). Profiles of proteins
Table 4-1. Percent incorporation of $[^3]$H]leucine into nondialyzable protein and protein content for the first, second, third and fourth 24 h culture periods of conceptuses.

<table>
<thead>
<tr>
<th>Culture Period</th>
<th>n</th>
<th>Percent incorporation</th>
<th>Protein content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>3</td>
<td>17.28 ± 11.67</td>
<td>2.16 ± 0.62</td>
</tr>
<tr>
<td>Second</td>
<td>2</td>
<td>49.66 ± 27.70</td>
<td>4.05 ± 2.17</td>
</tr>
<tr>
<td>Third</td>
<td>3</td>
<td>52.22 ± 20.50</td>
<td>3.03 ± 2.85</td>
</tr>
<tr>
<td>Fourth</td>
<td>3</td>
<td>13.02 ± 17.62</td>
<td>1.71 ± 0.15</td>
</tr>
</tbody>
</table>

$^a$ MEM and $[^3]$H]leucine were replaced every 24 h during culture.
Fig. 4-1. HPLC gel filtration profiles of radiolabelled conceptus-conditioned medium from the first, second, third, and fourth days of culture of a conceptus. Culture was performed in the presence of [³H]leucine. Conceptus supernatants were centrifuged (2,600 x g, 4°C) for 30 min to remove particulate matter and placed into dialysis tubing (Spectrum; 3,500 Mr cutoff) and dialysed extensively. Aliquots of medium containing approximately 25,000 dpm [³H]-leucine were injected onto a Zorbax GF-250 HPLC gel filtration column to resolve proteins. Note that unlike results of 2-D SDS-PAGE, bTP-1 is not the major molecule detected by HPLC. Protein standards were Thy = thyroglobulin, BSA = bovine serum albumin, CA = carbonic anhydrase and Myo = myoglobin.
present during the consecutive culture periods were similar, but some differences occurred. Of immediate interest is the fact that bTP-1, which migrated in fractions 42-44, did not represent the major conceptus product as observed in polyacrylamide gels. The percentage of radiolabel present in region 1 (fractions 28-35) which represents high molecular weight proteins (335 kDa; thyroglobulin) tended to increase from first to fourth 24 h period of culture (14.32%, 13.52%, 17.32%, and 17.98% of total counts recovered for first through fourth 24 h periods). A similar increase in proteins migrating in region 2 (fractions 36-40), the region of BSA (tube 37 = 69 kDa) was noted during the consecutive culture periods (10.9%, 11.98%, 13.86%, and 15.27% of recovered counts for each period). In contrast, proportion of counts recovered from region 3 (fractions 41-45) corresponding to bTP-1 elution (myoglobin = 18 kDa, tube 43; decreased with culture period (21.85%, 22.18%, 19.65%, and 19.66%, respectively) as did low molecular weight products (fractions 46-50; tube 50 = salt volume, 27.73%, 23.79%, 21.70%, and 21.75%, respectively). Although the elution of protein in the bTP-1 region did not decline dramatically during the culture period a small decrease (-2.2%) was noted.

**Purification**

Because bTP-1 has been identified as the complex of proteins secreted by bovine conceptuses, immunologically crossreactive with antiserum directed against oTP-1 (chapter 2), the first approach to purification was to construct an anti-oTP-1 affinity column. This method proved not to be feasible because of the relative low affinity
of rabbit anti-oTP-1 antibodies to bTP-1 (Vallet et al., 1988a) and due to crossreactivity of the antiserum with BSA (chapter 2). If 0.15 M salt was included in the affinity column loading buffer to reduce nonspecific binding, little or no binding of bTP-1 to the column occurred. Alternatively, nonspecific binding occurred if salt was not included in the loading buffer. It also was determined that bTP-1 was extremely sensitive to proteolytic cleavage. As monitored by HPLC gel filtration, radiolabelled proteins in fractions corresponding to bTP-1 complex decreased when conceptus culture supernatants were stored for several days at 4°C (data not shown). A protease inhibitor mixture of 1 mM EDTA, 0.7 mg/l pepstatin-A, and 0.5-mg/l of leupeptin or 0.2-mM PMSF was found to be effective in inhibiting proteolytic cleavage of proteins during storage and purification procedures carried out at 4°C.

The purification procedure utilized several well established methods. The first of these was saturated ammonium sulfate (SAS) precipitation of proteins. Aliquots of dialysed, lyophilized medium from conceptus culture with [35S]-methionine were precipitated with 40 or 50% SAS. Supernatants and precipitates were dialysed, concentrated, and protein distribution analysed by 1-D SDS-PAGE (Fig. 4-2, lane 6). Adding SAS to a final concentration of 50% (v/v) resulted in a majority of the bTP-1 complex remaining in solution while several other proteins, including some with molecular weights similar to bTP-1, were precipitated (Fig. 4-2). The process of SAS fractionation and dialysis resulted in a 4-fold increase in volume compared to the starting volume. Therefore had to devise a non-
Fig. 4-2. Fluorograph of electrophoretogram of bCSPs subjected to 40% or 50% saturated ammonium sulfate (SAS) precipitation. Lanes 1, 3, 4 and 5 represent material from bCSP found in the supernatant and lanes 2, 6, 7, and 8, material in the precipitate. Total material found in supernatant (lanes 1 and 3) and precipitates (lanes 2 and 6) are shown. Supernatants and precipitates were immunoabsorbed with rabbit anti-oTP-1 antiserum (lanes 4 and 7) or normal rabbit serum (lanes 5 and 8) as described in chapter 2.
denaturing and rapid method for concentration of sample before further purification. Supernatant was passed over CM-Sepharose equilibrated with buffer (pH 8.2) to remove basic proteins (pI>8.2). Anion-exchange chromatography was performed utilizing a DEAE-Sepharose column pH 8.2. Proteins with pI of 8.2 or lower bind, and theoretically, no protein is left in the material passing through the matrix. All potential protein of interest is recovered in a relatively small volume following elution from the DEAE matrix. Peak fractions were concentrated further by placing the material in dialysis tubing packed in Aquacide II. A small volume at this stage was critical, since 200 μl was the maximum volume separated during one injection on the HPLC system. Concentrated material was separated by HPLC gel filtration in 0.2 M NaPO₄, pH 7.9. Pooling of fractions was carried out after determining distribution of methionine radiolabelled bTP-1 complex and other proteins by 1-D SDS-PAGE of individual fractions (Fig. 4-3), and these were associated with chromatographic profiles of injection material. It is important to note that Fig. 4-3 represents methionine-labelled bCSP which had been partially purified, so labelling pattern may differ from patterns observed following isolation by other techniques. The region corresponding to bTP-1 (fractions 46-48) also contained a protein species at approximately 45 kDa which may represent an aggregate of bTP-1. This is supported by analysis of immunoblots described in this chapter. When immunoblot obtained by incubation with anti-oTP-1 antiserum was exposed to the point that the background became dark, crossreactive species migrating at 45 kDa was
Fig. 4-3. Fluorograph of electrophoretogram of bCSP after separation by HPLC gel filtration. Starting material for HPLC was bCSP that had been subjected to saturated ammonium sulfate precipitation and ion exchange chromatography. Conceptus proteins were obtained from the culture of day 17-18 conceptuses incubated in the presence of 100 μCi [35S]methionine for 24 h. Fractions (39-49) represent pools of 15 single injections (20 sec per fraction) which were subsequently dialysed, lyophilized and separated by 1-D SDS-PAGE.
observed (data not shown since autoradiograph could not be photographed). Aliquants of fractions were analyzed by liquid scintillation counting to make identification of the enriched bTP-1 containing fractions more reliable. The bTP-1 complex generally eluted in 3 to 5 fractions, and 3 to 4 of these were pooled for preparing infusates.

**Purification yield**

A total of 1.5 mg of purified bTP-1 was obtained through the processing of 73 culture equivalents containing 138.7 mg total protein. This represents a yield of 1.08% or 20 μg bTP-1 per culture equivalent of the starting material which is less than estimates of 5-10% of radiolabelled conceptus secretory components from the first 24 h of culture (Putney et al., 1988). This discrepancy is possibly the result of losses during purification or alternatively, that bTP-1 is less abundant in second and third 24 h culture supernatants.

Purity of bTP-1 was assessed by 2-D SDS-PAGE and silver staining (Fig. 4-4). Ten protein species could be visualized (Fig. 4-4, top left) including three at 22 kDa, five at 24 kDa and two at 26 kDa. This compares closely with results in which immunologically reactive components of the bTP-1 complex were first described (chapter 2). The only protein species detected by silver staining in the purified bTP-1 preparation that was not the result of nonspecific staining was a minor contaminant migrating as BSA (compare Fig. 4-4, top left to 4-4, top right, which represents a 2-D gel containing no protein).

Components in the sample loading buffer have been shown, as in Fig. 4-4 (top right), to cause nonspecific staining by the silver stain
Fig. 4-4. Silver-stained, two-dimensional electrophoretogram of highly purified bTP-1 complex (16 μg, top left panel); a gel which had been run with sample buffer, but no protein (top right panel); total array of bCSps (100 μg, bottom left panel) and bovine serum albumin (20 μg, bottom right panel).
method. Proteins present in bCSP and BSA preparations used for infusion are shown in Figure 4-4, bottom left and right, respectively. Immunoblotting using anti-oTP-1 antiserum was performed to verify that the purified proteins were bTP-1 (Fig. 4-5). The array of proteins that crossreacted with the antibody correspond exactly with those identified by silver staining in the purified bTP-1 preparation (Fig. 4-4 top left). In addition to these, a faint band at 45 kDa was visible in over-exposed autoradiographs indicating that aggregates of bTP-1 formed during purification. It appears that more isoelectric species of bTP-1 were identified as being immunoreactive in this study than those reported earlier (chapter 2). This may be due to enrichment of bTP-1 molecular components during purification that were not detectable by immunoprecipitation of bCSP as described earlier (chapter 2).

**Effects of intrauterine infusions on estrous cycle responses**

Treatment of cyclic cows with bTP-1 on days 15.5 to 21.0 extended the interestrous interval compared to control cows (Table 4-2). Orthogonal contrasts were made comparing 1) BSA vs bCSP and bTP-1 complex treatment and 2) bCSP vs bTP-1 complex treatment for all responses. Interestrous interval in which treatments were administered was greater (P<0.04) for cows treated with bCSP and bTP-1 complex than for BSA-treated cows. The increase was due to a longer interestrous interval for bTP-1 treated cows compared to bCSP- and BSA-treated cows (P<0.05).

**Progesterone profiles**

Differences in CL lifespan between groups was examined by evaluating plasma progesterone profiles by regression analysis in
Fig. 4-5. Autoradiograph of 2-D SDS-PAGE of purified bTP-1 complex (8 µg/gel) transferred to nitrocellulose and immunoblotted with a) rabbit anti-oTP-1 antiserum (1:100) or b) normal rabbit serum.
Table 4-2. Least square means ± sem for characteristics of estrous cycles for cows treated with BSA, bCSP or bTP-1 complex.

<table>
<thead>
<tr>
<th>Response</th>
<th>BSA</th>
<th>bCSP</th>
<th>bTP-1</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interestrous interval (a*, b*)</td>
<td>19.5</td>
<td>21.5</td>
<td>26.0</td>
<td>1.31</td>
</tr>
<tr>
<td>Interval to CL regression (b*2)</td>
<td>17.7</td>
<td>18.5</td>
<td>24.0</td>
<td>1.18</td>
</tr>
<tr>
<td>CL regression to estrus</td>
<td>1.8</td>
<td>3.2</td>
<td>2.0</td>
<td>0.54</td>
</tr>
<tr>
<td>Post-experimental interestrous interval</td>
<td>20.2</td>
<td>17.8</td>
<td>21.7</td>
<td>1.21</td>
</tr>
</tbody>
</table>

\* Contrast 1: BSA vs bCSP + bTP-1
\* Contrast 2: bCSP vs bTP-1
P<0.05
which treatment differences were tested by homogeneity of regression analysis (Knickerbocker et al., 1986b). Luteal phases were extended for bCSP and bTP-1 treatments compared to BSA treated controls (P<0.01; Figure 4-6). This difference was due largely to an extension of the luteal phase of bTP-1 treated cows compared to bCSP treated cows (P<0.01).

Orthogonal contrasts comparing the interval from pre-treatment estrus to when progesterone (P4) fell below 1 ng/ml was not significantly greater (P<0.1) for bCSP and bTP-1 vs BSA treated animals (18.5 and 24.0 vs 17.7 days, respectively), but was longer (P<0.02) for bTP-1 vs bCSP treated cows. The period between when P4 fell below 1 ng/ml and post-treatment estrus was not different between treatments (Table 4-2). Similarly, post-treatment estrous cycle lengths were not affected by treatments.

Prostaglandin profiles

Due to difficulties encountered with either cannulation or function of vena cava catheters, only two cows from each treatment group were sampled successfully by this method. Of these, both bTP-1 cows were sampled successfully on all sample days, while in the BSA cows, one animal was observed in estrus on the first day of sampling (day 19) and the other cow on the second day of sampling (day 20). Of the bCSP treated cattle, one was observed in estrus on the first day of sampling (day 19) and in the second, the catheter failed by the end of the first day of sampling (day 19). Due to poor representation of treatment groups on all days of sampling period, all sample values were analysed for treatment differences without
Fig. 4-6. Progesterone profiles of cattle receiving intrauterine infusion of bovine serum albumin (BSA, top panel); bCSP (middle panel) or purified bTP-1 complex (bottom panel) from day 15.5 to 21 of an estrous cycle.
Progesterone (ng/ml)

Day of estrous cycle

BSA TREATED COWS
- 201
- 296
- 311

bCSP TREATED COWS
- 289
- 241
- 317

bTFP-1 TREATED COWS
- 254
- 318
- 277
including day (19, 20, 21) in the model (treatment, cow within treatment and residual were considered in the model).

Residual variances for individual treatments are shown in Table 4-3. Variances for bTP-1 treatment cows were lower than for bCSP and BSA treated cows which was apparent when examining individual profiles of cows sampled on each day. The BSA treated cows exhibited elevations of plasma PGF concentrations, whereas PGF for bTP-1 and most bCSP treated animals was undetectable by radioimmunoassay (Fig. 4-7). Due to heterogeneity of variances, data were transformed by examining the reciprocal of PGF values for statistical analysis. Transformation reduced heterogeneity of variance, but variances were still different (P<0.05). Analysis of variance to test treatment means indicated that the BSA versus bTP-1 + bCSP contrast approached significance (P<0.1). Concentrations of PGF for BSA treated cows was nearly twice that for bTP-1 and bCSP cows (Table 4-3). The fact that residual variance and mean PGF secretion for bTP-1 and bCSP cows were lower than for BSA groups is of biological significance. Components of variation in residual variances for each treatment include experimental error, but also sample to sample variation among the consecutive 25, 15 min periods within and among the days for each of the cows. This type of variation would be associated with the pulsatile nature of IGF secretion. Reduction in mean concentration and residual variance indicated that bTP-1 and bCSP reduced secretion by the uterus compared to BSA (Table 4-3).
Table 4-3. Least squares means ± sem of plasma PGF concentrations and residual variances for BSA, bCSP and bTP-1 treated cattle.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BSA</th>
<th>bCSP</th>
<th>bTP-1</th>
</tr>
</thead>
</table>
| Plasma PGF (ng/ml)
| 97.13 | 56.82| 53.28 |
| SEM             | 9.79 | 2.69 | 1.13  |
| Residual variance | 7,199.08 | 326.42 | 192.02 |

*orthogonal contrast: BSA vs bCSP +bTP-1 (P<0.1).*
Fig. 4-7. Representative profiles for plasma PGF of a BSA, bCSP, and bTP-1 treated cow sampled every 15 min on Day 19 after estrus. Sensitivity of the assay was 50 pg/ml.
Infusion of bTP-1 was effective in extending CL function in this study. In contrast, bCSP treatment had little effect upon interesting intervals or CL function as monitored by progesterone secretory profiles. This latter result is in contrast to those of Knickerbocker et al. (1986b) in which intrauterine infusion of bCSP extended luteal lifespan. Knickerbocker and coworkers utilized only conceptus secretory proteins from the first 24 h of incubation. The present study utilized a pool of conceptus secretory proteins from the first 72 h of culture. Although the amount of bTP-1 in each of these consecutive culture periods was not quantified, it is possible that secretion of bTP-1 from conceptuses during the second and third 24 h periods of culture may have been lower than during the first 24 h. If bTP-1 present in bCSP was less than normally seen by the uterus during pregnancy, the treatment may not have been as effective in extending the interesting interval due to an inadequate triggering of the antiluteolytic effect. In contrast, adequate quantities of bTP-1 obtained from purification was infused into cows as a treatment that did extend CL lifespan. An additional advantage may have been given to the bTP-1 treatment group. The bCSP treatment group received 1.5 mg bCSP per day. This represented 79% of the protein secreted for one culture equivalent (1.9 mg), and was used so as to be consistent with infusion amounts administered by Knickerbocker et al. (1986b). However, bTP-1 treated cows received, during each day, bTP-1 which had been purified from 4.11 conceptus-culture equivalents. Thus the bTP-1 treated cows were given considerably more
bTP-1 complex proteins than cows receiving unfractionated bCSP. This is speculative since losses of bTP-1 during purification are not known. The bTP-1 content of second and third 24 h culture periods was also not quantified and could have been a factor in the efficacy of bCSP treatment.

The cycle extension brought about by bTP-1 treatment appears to be due to a direct effect of increasing length of the luteal phase, as shown by an increase in period from pretreatment estrus to when P₄ fell below 1 ng/ml. This extension was not due to an increase in the follicular phase since the period from when P₄ fell below 1 ng/ml to post-treatment estrus was not different between treatment groups. Rescue of the CL could be the result of several possible mechanisms. It has been well established that luteolysis occurs via secretion of PGF-2 from the uterus of cattle (Wolfenson et al., 1987). There is attenuation of PGF release from cultured (Thatcher et al., 1984b; Gross et al., 1988a) or perifused (Gross et al., 1988b) endometrial tissues of pregnant cattle compared to cyclic cattle. Knickerbocker et al. (1986a,b,) demonstrated that cattle which received intrauterine infusions of bCSP had extended CL function and attenuated PGF secretion and release in response to an estradiol challenge. In this experiment, bTP-1 appeared to inhibit PGF release, allowing extended CL maintenance. The exact mechanism whereby bTP-1 attenuates PGF release is not known. It has been demonstrated that endometrial inhibitors to prostaglandin synthesizing enzymes exist and their activity is increased during pregnancy (Basu & Kindahl, 1987; Gross et al., 1988b). Furthermore,
bCSP induces prostaglandin inhibitor activity (Gross et al., 1988a). It seems possible that bTP-1 which has now been identified as the bovine conceptus antiluteolytic signal, attenuates uterine PGF release by induction of inhibitors to prostaglandin synthesizing enzymes.
CHAPTER 5
BOVINE TROPHOBLAST PROTEIN-I COMPLEX ALTERS ENDOMETRIAL PROTEIN AND PROSTAGLANDIN SECRETION AND INDUCES AN INTRACELLULAR INHIBITOR OF PROSTAGLANDIN SYNTHESIS IN VITRO.

Introduction

Continued secretion of P4 by the CL is essential for maintenance of early pregnancy in the cow. Since PGF-2α from the uterine endometrium initiates regression of the CL in the absence of pregnancy (see Thatcher et al., 1984a), the conceptus must attenuate PGF secretion if pregnancy is to be maintained. This concept is supported by several lines of evidence. During the estrous cycle, episodic release of 13,14-dihydro-15-keto-PGF-2α (PGFM) is associated with declining progesterone concentrations (Thatcher et al., 1986b; Helmer & Britt, 1987) and presence of a viable embryo abolishes episodic PGFM release (Kindahl et al., 1976; Betteridge et al., 1984). Furthermore, release of PGFM in response to exogenous oxytocin is decreased during early pregnancy compared to the estrous cycle (Lafrance & Goff, 1985). The in vitro secretion of PGF also is decreased in cultured (Thatcher et al., 1984b; Gross et al., 1988a) or perfused (Gross et al., 1988b) endometrium from day 17 of pregnancy compared to day 17 of the estrous cycle.

The conceptus exerts its effects through release of secretory macromolecules. Intrauterine infusion of total pattern of secretory proteins from day 17 conceptuses (bCSP) into cyclic cattle was
associated with decrease in the episodic release of PGF (Knickerbocker et al., 1986b) and an extension of the interestrous interval. In cyclic cattle, injection of estradiol-17β will induce release of PGF-2α from the uterus (Knickerbocker et al., 1986c; Thatcher et al., 1986b), and intrauterine infusion of bCSP from day 15 to 18 after estrus will attenuate this release (Knickerbocker et al., 1986a). Treatment of endometrial explants cultured with bCSP also reduced PGF secretion in vitro (Gross et al., 1988a).

It was hypothesized by Thatcher et al. (1985) that secretory proteins of the conceptus stimulate an endometrial protein that inhibits PGF-2α synthesis. Evidence has accumulated to support this concept: a protein in endometrial homogenates from cyclic cows can inhibit prostaglandin generating enzymes. This activity is increased during early pregnancy (Basu & Kindahl, 1987; Gross et al., 1988b), and can be enhanced in endometrial explants by culture with bCSP.

Immunohistochemical localization (Godkin et al., 1984a) and receptor binding (Stewart et al., 1988) studies have shown that oTP-1, the anti-luteolytic hormone of the sheep conceptus, is present in epithelial cells lining the endometrium. Qualitative differences in the secretion of proteins from cyclic endometrial tissues cultured in the presence or absence of oTP-1 have been reported for sheep (Godkin et al., 1984a; Vallet et al., 1987). Differences in secretory profiles of uterine flushings (Bartol et al., 1981b) and endometrial secretory proteins (Geisert et al., 1988; Gross et al., 1988a) from cyclic and pregnant cattle have also been identified. Furthermore, proteins released by cultured endometrium from cyclic cows can be
altered qualitatively by incubation with bCSP (Gross et al., 1988a). In cattle, the bTP-1 complex is a group of isomers of N-linked glycoproteins in two size classes (22 kDa high-mannose type and 24 kDa complex-type) that are immunologically related to oTP-1 (chapters 2 and 3). This complex of proteins appears to be the conceptus signal that blocks luteolysis during early pregnancy because intrauterine infusion of bTP-1 delayed luteolysis (chapter 4). The objective of these experiments was to determine whether 1) bTP-1 is the component of conceptus secretions that is responsible for altered endometrial prostaglandin and protein secretions, and 2) bTP-1 stimulates production of the endometrial intracellular inhibitor of prostaglandin synthesis.

Materials and Methods

Materials

Radioisotopes of L-[4,5-3H]leucine (specific activity: 150 μCi/mmol), [5,6,8,11,12,14,15-3H]PGF-2 (specific activity: 160-180 μCi/mmol) and [5,6,8,12,14,15-3H]PGE-2 (specific activity: 140-170 μCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL.). Arachidonic acid was from Sigma Chemical Company (St. Louis, MO.). Tissue culture supplies were purchased from vendors as noted in chapter 2. Aquacide II (sodium carboxymethylcellulose) was purchased from Calbiochem, San Diego, CA. All other chemicals were reagent grade or better.
In Vitro Culture of Conceptuses

Beef cattle (Angus and Brangus) were detected in estrus, bred by natural service, slaughtered on day 17 or 18 after estrus (estrus = Day 0), and conceptuses recovered as previously described (chapter 2). Conceptuses were cultured for 72 h with medium, prepared as described in chapter 2, being replaced every 24 h (for conditions see chapter 4). Cultures were carried out in either complete MEM or in leucine-deficient (0.1 x) MEM supplemented with 0.1 mCi [3H]leucine/culture/24 h. Culture supernatants were frozen at -20°C until utilized.

Preparation of Samples for Incubation with Endometrium

Total bCSP from the first, second and third 24 h of culture were pooled and dialyzed against 10 mM sodium phosphate, pH 7.4 with 0.9% NaCl and concentrated by placing the sample in dialysis tubing packed in Aquacide II, as described in chapter 4. This resulted in a final concentration of 975 μg/ml. The amount of total protein from a culture equivalent (chapter 4) was approximately 1.9 mg/culture following dialysis and concentration. Endometrial culture in the presence of bCSP was carried out with an amount of bCSP equivalent to 10% of a culture equivalent (190 μg bCSP in 195 μl buffer).

The bTP-1 complex was purified as described in chapter 4. A stock concentration of 15 μg bTP-1/ml in 0.2 M NaPO₄, pH 7.4, was prepared and utilized at a final concentration of 1 μg/ml in medium for incubation with endometrium. Both bCSP and bTP-1 solutions were filter-sterilized (0.2 μm) prior to addition to incubations.
In Vitro Culture of Endometrial Tissues

Brangus cows (n=4) were observed for estrus and slaughtered on day 17 after estrus (estrus = day 0). Reproductive tracts were recovered; endometrium from the uterine horn ipsilateral to the CL was isolated from myometrium, cut into 1-3 mm³ pieces and cultured. For each cow, the following cultures were prepared in duplicate in 100 mm petri dishes containing 500 mg tissue: 1) BSA: 120 μg/ml (22.8 mg BSA added to 190 ml MEM), 24 μg/ml (23 ml of the 120 μg/ml solution added to 92 ml MEM), 4.8 μg/ml (20 ml of the 24 μg/ml stock solution added to 80 ml MEM) and 0 μg/ml (no BSA added) (14 ml of each stock and 1 ml 0.2 M NaPO₄, pH 7.4); 2) bCSP: 12.7 μg/ml (195 μl bCSP [190 μg] mixed with 800 μl 0.2 M NaPO₄ and 14 ml MEM); or 3) bTP-1: 1 μg/ml (1 ml of bTP-1 [15 μg] in 0.2 M NaPO₄ mixed with 14 ml MEM). Each culture also included addition of arachidonic acid (0.2 mg in 0.1 ml MEM). Samples of medium (0.5 ml) were removed at 6, 12, 18 and 24 h of incubation. An additional set of cultures was prepared from each cow except that 250 mg of tissue was incubated in 60 mm petri dishes in duplicate; one half the tissue weight, medium volume and arachidonic acid dose, as used in the previously described cultures, were utilized per dish. These incubations were carried out in leucine deficient (0.1 x) MEM supplemented with 50 μCi L-[4,5-³H] leucine. Samples of medium (0.25 ml) were taken at 6, 12, 18 and 24 h of incubation. At the end of incubations, tissue and medium were separated by centrifugation (3,500 x g, 30 min, 4°C), and each stored at -20°C until analyzed. In all cases, incubations were carried out on a rocking platform at 39°C for 24 h in an atmosphere of 47.5%
on a rocking platform at 39°C for 24 h in an atmosphere of 47.5% oxygen, 2.5% carbon dioxide and 50% nitrogen (v/v).

Assay for Inhibition of Prostaglandin Synthesis

Cotyledonary microsomes prepared from parturient cattle and endometrial cytosolic supernatant from endometrial explants (100,000 x g) were prepared as described previously (Gross et al., 1988b). Cytosolic supernatants from endometrial cultures were obtained by pooling tissue from duplicate cultures (1 g total weight) and reconstituting the high speed supernatant to 1 ml with potassium phosphate buffer, pH 7.5. Cotyledonary microsomes from parturient cattle were utilized as a prostaglandin-generating system for the detection of inhibitors to PG synthesis. The generating system (0.5 ml; 500 mg tissue equivalent) was incubated with 0.1 mg arachidonic acid (0.1 ml) and with either 0.1 M potassium phosphate buffer (0.4 ml, pH 7.5) or endometrial cytosolic supernatant (0.4 ml; 400 mg tissue equivalent). Final incubation volume was brought to 2 ml with 0.1 M potassium phosphate buffer, pH 7.5. Incubations were conducted for 1 h at 39°C on a rocker platform. Incubations were terminated by addition of 0.25 ml ethanol. Samples were centrifuged (1,500 x g) for 20 min at 4°C to pellet the ethanol-precipitated material. Supernatants were analyzed directly for PGF using radioimmunoassay (Gross et al., 1988b).

Radiolabeled Protein Incorporation

Incorporation of radiolabelled leucine into secreted (medium) and intracellular (tissue) proteins was determined by trichloroacetic acid (TCA) precipitation. Tissue from incubations with [³H]leucine
was solubilized in 50 mM Tris-acetate buffer (2 ml buffer/500 mg tissue) containing 1 mM phenylmethylsulfonyl-flouride, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% (v/v) Nonidet P-40. Duplicate aliquots (0.05 ml) of medium and solubilized tissue each were placed and dried onto Whatman 3MM paper that previously had been saturated with 20% TCA (wt/vol). Precipitation of proteins onto the filter paper and removal of nonproteinaceous compounds was accomplished by serial washing of filter paper with 20% and 5% TCA followed by 95% ethanol as described by Mans and Novelli (1961). Radioactivity of precipitated protein was determined by scintillation spectrometry.

Radioimmunoassay Procedures

Single determinations with replication of every fifth sample for PGF and PGE concentrations were made. Samples of medium were assayed for PGF by radioimmunoassay (RIA) procedures described by Knickerbocker et al. (1986c). The assay was modified to use an antibody supplied and characterized by Kennedy (1985), and crossreactivities of the PGF antiserum as well as assay validation were described previously by Gross et al. (1988a). Crossreactivity of the PGF antiserum for PGF-1 was 94%, so results of assays performed with this antiserum are referred to as PGF activity. Crossreactivities of the PGF antiserum with other PGs were 2.4% for PGE-2; < 0.1% for 13,14-dihydro-15-keto-PGF-2α, PGE-1, 6-keto-PGF-1 and arachidonic acid. Interassay and intrassay coefficients of variation were 11.3% and 12.4%, respectively.
A procedure to measure PGE-2 was utilized with modifications from an earlier RIA procedure and an antibody characterized by Lewis et al. (1978). The assay was validated and cross-reactivities described by Gross et al. (1988a). Cross-reactivities of the PGE-2 antiserum with PG were: 24% for PGE-1; 1.7% for PGF-2α; < 0.1% for 13,14-dihydro-15-keto-PGF-2α, PGF-1, 6-keto-PGF-1α and arachidonic acid. Interassay and intrassay coefficients of variation were 9.8% and 13.2%, respectively.

**Statistical Analyses**

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Data on incorporation of radiolabel into secreted proteins were analyzed using the model components of treatment (medium, BSA, bCSP, bTP-1), cow, treatment x cow, replicate within (treatment x cow), time (6, 12, 18, 24 h), treatment x time, cow x time, treatment x cow x time, time x replicate within (treatment x cow) and residual (Table 5-1). Incorporation of radiolabel into tissue was analyzed using the model components of treatment, cow, treatment x cow, replicate within (treatment x cow) and residual (Table 5-1). Treatments were compared by using orthogonal contrasts: 1) Medium vs BSA + bCSP + bTP-1, 2) BSA vs bCSP + bTP-1, and 3) bCSP vs bTP-1. Final models for analysis of PGF and PGE secretory responses were the same as for incorporation of radiolabel into medium except that 1) treatments compared were BSA, bCSP and bTP-1, and 2) time x replicate within (treatment x cow) was excluded from the model (Table 5-2). Treatments were compared by
Table 5-1. Analysis of variance for incorporation of $[^{3}\text{H}]$-leucine into secretory proteins by endometrial explants treated with BSA, bCSP or bTP-1 complex.

<table>
<thead>
<tr>
<th>Source</th>
<th>Medium</th>
<th></th>
<th>Tissue</th>
<th></th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>SS ($\times 10^{-12}$)</td>
<td></td>
<td>SS ($\times 10^{-12}$)</td>
<td>term</td>
</tr>
<tr>
<td>trt$^a$</td>
<td>3</td>
<td>3.733***</td>
<td>216,479***</td>
<td>T x C</td>
<td></td>
</tr>
<tr>
<td>media vs BSA+bCSP+bTP</td>
<td>1</td>
<td>0.199</td>
<td>5.752</td>
<td>T x C</td>
<td></td>
</tr>
<tr>
<td>BSA vs bCSP+bTP</td>
<td>1</td>
<td>3.522***</td>
<td>210,518***</td>
<td>T x C</td>
<td></td>
</tr>
<tr>
<td>bCSP vs bTP</td>
<td>1</td>
<td>0.012</td>
<td>0.210</td>
<td>T x C</td>
<td></td>
</tr>
<tr>
<td>cow</td>
<td>3</td>
<td>5.958***</td>
<td>158.555***</td>
<td>R(T x C)</td>
<td></td>
</tr>
<tr>
<td>trt x cow</td>
<td>9</td>
<td>0.459**</td>
<td>34.149***</td>
<td>R(T x C)</td>
<td></td>
</tr>
<tr>
<td>rep(trt x cow)</td>
<td>16</td>
<td>0.189***</td>
<td>10.912***</td>
<td>residual</td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>3</td>
<td>9.074***</td>
<td></td>
<td>C x Ti</td>
<td></td>
</tr>
<tr>
<td>trt x time</td>
<td>9</td>
<td>1.650***</td>
<td></td>
<td>T x C x Ti</td>
<td></td>
</tr>
<tr>
<td>cow x time</td>
<td>9</td>
<td>2.198***</td>
<td></td>
<td>residual</td>
<td></td>
</tr>
<tr>
<td>trt x cow x time</td>
<td>27</td>
<td>1.079***</td>
<td></td>
<td>residual</td>
<td></td>
</tr>
<tr>
<td>time x rep(trt x cow)</td>
<td>48</td>
<td>0.229</td>
<td></td>
<td>residual</td>
<td></td>
</tr>
<tr>
<td>residual</td>
<td>128</td>
<td>0.428</td>
<td>3.440</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** P<0.01.

*** P<0.005.

$^a$ Definitions: T, trt, treatment; C, cow; R, rep, replications; Ti, the
Table 5-2. Analysis of variance for PGF and PGE in medium for endometrial explants incubated with BSA, bCSP or bTP-1.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>PGF</th>
<th>PGE</th>
<th>Error term</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>df</td>
<td>SS</td>
<td></td>
</tr>
<tr>
<td>trt</td>
<td></td>
<td></td>
<td>136,125***</td>
<td>20,379***</td>
</tr>
<tr>
<td>BSA vs bCSP+bTP</td>
<td>1</td>
<td>136,017***</td>
<td>925</td>
<td>T x C</td>
</tr>
<tr>
<td>bCSP vs bTP-1</td>
<td>1</td>
<td>363</td>
<td>19,642***</td>
<td>T x C</td>
</tr>
<tr>
<td>cow</td>
<td>3</td>
<td>61,101***</td>
<td>4,079</td>
<td>R(T x C)</td>
</tr>
<tr>
<td>trt x cow</td>
<td>6</td>
<td>9,886</td>
<td>2,227</td>
<td>R(T x C)</td>
</tr>
<tr>
<td>rep(trt x cow)</td>
<td>12</td>
<td>9,814</td>
<td>6,645</td>
<td>residual</td>
</tr>
<tr>
<td>time</td>
<td>3</td>
<td>781,751***</td>
<td>267,298***</td>
<td>C x Ti</td>
</tr>
<tr>
<td>trt x time</td>
<td>6</td>
<td>3,485</td>
<td>9,524***</td>
<td>T x C x Ti</td>
</tr>
<tr>
<td>cow x time</td>
<td>9</td>
<td>6,879</td>
<td>3,414</td>
<td>residual</td>
</tr>
<tr>
<td>trt x cow x time</td>
<td>18</td>
<td>15,205</td>
<td>4,612</td>
<td>residual</td>
</tr>
<tr>
<td>residual</td>
<td>35</td>
<td>23,591</td>
<td>12,702</td>
<td></td>
</tr>
</tbody>
</table>

*** P<0.005.
using orthogonal contrasts: 1) BSA vs bCSP + bTP-i, and 2) bCSP vs bTP-i. Results on inhibition of prostaglandin synthesis in the prostaglandin generating system by cytosols from explant tissues were analyzed using the model components of cow, treatment and error. Treatments were compared by orthogonal contrasts: 1) generating system vs BSA + bCSP + bTP-i, 2) BSA vs bCSP + bTP-i, and 3) bCSP vs bTP-i.

Results

Prostaglandin Secretion by Endometrial Explants

Prostaglandin secretion by endometrial tissue explants (day 17) treated with 0, 4.8, 24 or 120 μg BSA per ml were not different from each other (PGF: 482, 494, 481 and 478 ± 27 ng/ml; PGE-2: 209, 198, 206 and 193 ± 9 ng/ml, respectively). The 4.8 μg BSA per ml treatment group was used for control comparisons in subsequent analyses because this concentration of BSA most closely approximated that of bCSP (12.7 μg/ml) and bTP-i (1 μg/ml) treatment groups. Secretion of PGF by endometrial explants from cyclic cattle (Table 5-3) increased with time (P<0.01), however, secretion was decreased by bCSP and bTP-i treatments as compared to BSA controls (P<0.005). The bCSP and bTP-i induced reduction of PGF secretion occurred during the first 6 h of incubation. During the remaining period from 6 to 24 h the rate of increase was the same between BSA, bCSP and bTP-i treatment groups. The parallel responses between treatments is supported by the non-significant interaction of treatment by time (Table 5-2). However, the main affect of treatment was detected
Table 5-3. Least squares means\(^a\) of concentrations of PGF and PGE secreted into medium of day 17 endometrial explants incubated for 24 h with medium containing 4.8\(\mu\)g BSA/ml, 12.7 \(\mu\)g bcSP/ml or 1 \(\mu\)g bTP-1/ml.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Time (h)</th>
<th>BSA</th>
<th>bcSP</th>
<th>bTP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF</td>
<td>6</td>
<td>240.5</td>
<td>171.5</td>
<td>163.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>315.1</td>
<td>245.6</td>
<td>241.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>380.8</td>
<td>309.8</td>
<td>295.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>508.8</td>
<td>406.4</td>
<td>414.5</td>
</tr>
<tr>
<td>PGE</td>
<td>6</td>
<td>64.3</td>
<td>64.5</td>
<td>71.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>101.5</td>
<td>91.8</td>
<td>109.8</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>151.5</td>
<td>136.6</td>
<td>177.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>199.1</td>
<td>178.5</td>
<td>251.0</td>
</tr>
</tbody>
</table>

\(^a\)Pooled sem for PGF and PGE are 10.28 and 5.66ng/500 mg/15 ml, respectively.
due to a lower mean secretion of PGF by bCSP and bTP-1 treatments during the first 6 h period. Secretion of PGF did not differ between bCSP and bTP-1 treated explants. Comparison of secretion of PGE-2 by endometrial explants treated with bCSP and bTP-1 to that secreted by BSA-treated explants revealed no significant effect. Differences in PGE secretion were detected between treatments (Table 5-2) in which bTP-1 stimulated and bCSP reduced secretion of PGE (P<0.01). This differential response was such that the bTP-1 and bCSP treatments together were not different from BSA but were different from each other. Furthermore, mean PGE secretion by explants treated with bTP-1 was greater (P<0.005) than by those treated with bCSP (Table 5-3). A significant treatment x time interaction was detected due to an amplification of PGE-2 endometrial secretion between 12 and 24 h in response to bTP-1 (P<0.005) and a decrease in response to bCSP.

**Induction of an Intracellular Inhibitor of Prostaglandin Synthesis**

Prostaglandin synthesis by the generating system was decreased slightly (P<0.05) by cytosol from explants treated with 4.8 µg BSA/ml (9%), whereas cytosol from bCSP and bTP-1 treated explants markedly decreased (P<0.01) PGF synthesis (42 and 35%, respectively; Fig. 5-1). The reduction in prostaglandin synthesis by the generating system caused by adding cytosol from explants treated with bCSP was not significantly different from the inhibition caused by cytosol from bTP-1 treated tissue.
Fig. 5-1. Least squares means (pooled sem=0.20) for PG synthesis by the prostaglandin generating system in the presence of cytosolic supernatants from day 17 endometrial explants which had been treated with no BSA, 4.8 μg BSA/ml, 12.7 μg bCSP/ml or 1 μg bTP-1/ml for 24 h.
Incorporation of Radiolabel into Macromolecules

For all treatments, accumulated incorporation of radiolabel into secreted proteins increased (P<0.01) with time of incubation. Incubation of endometrial explants with 4.8, 24 or 120 μg BSA/ml increased (P<0.01) incorporation of radiolabeled precursors into secreted (0.96, 0.97 and 1.03 ± 0.05 dpm x 10^-6/250 mg/24 h, respectively) and tissue proteins (6.54, 6.39 and 6.50 ± 0.79 dpm x 10^-6/250 mg/24 h, respectively) compared to explants not treated with BSA (0.30 ± 0.05 dpm x 10^-6/250 mg/24 h and 1.84 ± 0.79 dpm x 10^-6/250 mg/24h, respectively). The effect of BSA was not concentration dependent, thus 0 and 4.8 μg/ml BSA doses were utilized as controls to compare to other treatment responses. Treatment of endometrial explants from cyclic cows with bCSP or bTP-1 decreased (P<0.01) incorporation of radiolabel into secreted proteins compared to treatment with 4.8 μg/ml BSA (51.7% and 56.2% reduction at 24 h, respectively; Table 5-4). This effect also caused a treatment x time interaction (P<0.01). Incorporation rates were not different when comparing explants treated with bCSP and bTP-1 to explants treated without any BSA (9.0 and 17.7% reduction at 24 h, respectively; Table 5-4).

Incorporation of radiolabel into tissue proteins at the end of the 24 h incubation (Table 5-4) was decreased (P<0.01) for bCSP and bTP-1 treated explants compared to explants treated with 4.8 μg/ml BSA (P<0.01; 64.2 and 66.6% reduction at 24 h, respectively) and when compared to explants treated without BSA (P<0.10, 22.5 and 27.7% reduction at 24 h, respectively).
Table 5-4. Least squares means\(^a\) for incorporation of \(^{3}\text{H}\)-leucine into TCA-precipitable macromolecules in medium (dpm/250 mg/15 ml) and tissue (dpm/250 mg/24h) from endometrial explants incubated for 24 h with medium containing no BSA, 4.8 \(\mu\)g BSA/ml, 12.7 \(\mu\)g bCSP/ml or 1 \(\mu\)g bTP-1/ml.

<table>
<thead>
<tr>
<th>Source</th>
<th>Time (h)</th>
<th>Medium</th>
<th>BSA</th>
<th>bCSP</th>
<th>bTP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted</td>
<td>6</td>
<td>1.255</td>
<td>1.566</td>
<td>1.041</td>
<td>1.084</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.144</td>
<td>3.765</td>
<td>2.263</td>
<td>1.708</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>3.644</td>
<td>7.236</td>
<td>3.355</td>
<td>3.556</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.346</td>
<td>10.063</td>
<td>4.865</td>
<td>4.403</td>
</tr>
<tr>
<td>Tissue</td>
<td>24</td>
<td>31.390</td>
<td>67.930</td>
<td>24.314</td>
<td>22.695</td>
</tr>
</tbody>
</table>

\(^a\) Pooled sem for TCA-precipitable macromolecules into secreted and tissue proteins was 0.707 and 6.887, respectively.
Discussion

Results from the present experiment support the hypothesis (Thatcher et al., 1985) that bCSP causes maintenance of the CL by inducing an inhibitor of the prostaglandin-synthesizing enzymes. The induction of the prostaglandin synthesis inhibitor seen in the present experiment likely accounted for the decrease in secretion of PGF caused by bCSP and bTP-1. This response is in agreement with results of Gross et al. (1988a) showing culture of endometrium from cyclic cows with bCSP reduced PGF secretion and enhanced activity of the prostaglandin synthesis inhibitor. The present results indicate that bTP-1 likely was an active component of bCSP in the experiment of Gross et al., (1988a) and the current experiment. Likewise, in vivo results indicated that intrauterine infusion of bCSP reduced PGF secretion (Knickerbocker et al., 1986a,b). It is likely that bTP-1 is an active component of bCSP regulating PGF secretion by the endometrial tissue. This is further confirmed by the in vivo experiment in which intrauterine infusion of bTP-1 extended interestrous interval and attenuated PGF secretion (chapter 4). Of interest in the present study was the dynamics of PGF secretion during culture of endometrial explants treated with BSA, bCSP and bTP-1 for 24 h. Both bCSP and bTP-1 decreased PGF secretion, the suppression being manifested during the first 6 h and rate of secretion thereafter appeared to be similar between treatments. Reduction in PGF secretion is likely to be partially due to induction of a PGF inhibitor since inhibitor activity was increased in tissues assayed at the end of 24 h. In fact, percent inhibition of PGF
synthesis in the PG-generating system was 34.8% and 25.4% for bCSP and bTP-I treatments, which approximates the mean percent inhibition of PGF secretion by endometrial explants treated with bCSP (21.6%) and bTP-I (23.0%) during the 24 h culture period. A reasonable question is why wasn't the inhibition of PGF secretion sustained between 6 and 24 h of culture. Several possibilities could account for this effect. A certain amount of the inhibitor was induced by both bCSP and bTP-I, but continued production of additional inhibitor was not sustained due to the breakdown of essential bCSP components such as bTP-I during the 24 h period at 37°C. When examining the kinetics of the cyclooxygenase generating system of parturient cotyledons, in response to the inhibitor in high speed cytosol of day 17 pregnant tissues, it was determined that inhibitor was noncompetitive in nature relative to arachidonic acid substrate (Gross et al., 1988b). Addition of arachidonic acid to the generating system plus inhibitor increased PGF secretion, but less than at lower doses of the inhibitor. In an intact endometrial explant system comprised of various cell types (eg. epithelium and stromal cells), the uptake of arachidonic acid from the medium and its entrance into the phospholipid pool for subsequent synthesis of prostaglandins is likely to increase with time. Consequently, the degree of inhibition may not be sustained throughout the culture period in the presence of a fixed amount of inhibitor. Such effects that may be occurring in certain cell types and not others may have precluded the detection of a sustained attenuation in PGF secretion throughout the 24 h period. Nevertheless, removal of the high speed
cytosol from the endometrial explants at the termination of the culture and its addition to a cell free cyclooxygenase system caused suppression in PGF production that was greater in preparations from bCSP and bTP-1 treated tissues compared to BSA treatment. An alternative explanation for not detecting differences in PGF secretion between treatments after 6 h is that the frequency of sampling was not sufficient to characterize the response. Additional studies by Gross et al. (personal communication) indicate that treatment of endometrial explants with bCSP results in a reduction in the secretion rates of PGF during a 12 h period when media was sampled at 4, 6, 8, 10 and 12 h.

Of interest in the present study was a differential effect of bTP-1 on secretion of PGF and PGE-2 by endometrial explants. While bTP-1 lowered secretion of PGF, as did bCSP, the secretion of PGE-2 by explants treated with bTP-1 was enhanced compared to bCSP. It is unlikely that the increase in PGE-2 and decrease of PGF is due to insufficient arachidonic acid substrate. In either absence or presence of arachidonic acid, clear differences are detected in PGF secretion between cyclic and pregnant endometrial tissue (cyclic > pregnant), whereas PGE-2 secretion did not vary between tissue (Thatcher et al., 1984a). Likewise, in the present study, exogenous arachidonic acid (200 µg) was added to medium and products of the conceptus (bCSP and bTP-1) decreased PGF secretion compared to BSA control treatment. However, bTP-1 enhanced PGE-2 secretion. This implies that some other component of bCSP inhibits PGE-2 secretion and that bTP-1 exerts differential regulatory effects on PGF and PGE-
secretion in endometrial explants. Endometrial explants are composed of different cell types (e.g., luminal epithelium and stromal cells), and bTP-1 is composed of two molecular weight glycoprotein classes (22 and 24 kDa), each with several isoelectric variants (chapter 3; Anthony et al., 1988). Consequently, either differential regulation by the molecular weight components or differential response of cell types may influence the type of prostaglandin secretion in response to bTP-1. Fortier et al. (1988) demonstrated that PGF is secreted primarily by epithelial cells of bovine endometrium, whereas PGE-2 is secreted primarily by stromal cells. Therefore, one major action of bTP-1 would appear to be on epithelium since PGF secretion was inhibited via induction of an inhibitor to prostaglandin synthesis. Since PGE-2 production by the epithelium is small compared to that from stroma, then it would not be unexpected that bTP-1 would have a minor effect on the net secretion of PGE-2 by the endometrium. Collectively, these data suggest that the intracellular endometrial prostaglandin synthesis inhibitor is present in the epithelium rather than the stromal cells of the endometrium. It is also possible that bTP-1 may act to shift production of PGF to PGE-2 or to activate enzymes to convert PGF to PGE-2, since PGE-2 tended to increase after PGF was decreased. Future studies are needed to examine the effects of bTP-1 on isolated bovine epithelial and stromal cells of the uterus during the estrous cycle and pregnancy. Differential regulation of PGF and PGE-2 secretion is supported by endometrial perifusion studies of Gross et
in which PGF secretion was reduced in pregnant endometrial tissues but PGE-2 secretion was not.

Incorporation of $[^3\text{H}]$leucine into endometrial secretions and tissue proteins was reduced by bCSP and bTP-1 as compared to BSA treated tissues. The reduction in incorporation of radiolabel into tissue proteins indicated that synthesis and secretion were reduced and agrees with results of Gross et al., (1988a). This reduction in incorporation would appear to represent a mechanism whereby the developing conceptus directly influences the endometrial synthesis of protein during pregnancy which confirms the finding that the uterine endometrium appears to be a primary target of bTP-1. Furthermore, bTP-1 acts on endometrium to attenuate secretion of PGF through activation of an inhibitor to prostaglandin synthesis enzymes. This ensures continued CL function and contributes to successful establishment of pregnancy. Thus, conceptus proteins appear to act on endometrium to alter the environment in which the conceptus resides and to ensure continued lifespan of the CL by inducing proteinaceous regulators of prostaglandin synthesis.
CHAPTER 6
GENERAL CONCLUSIONS

Results accumulated during the last 20 years have ascribed conceptus proteins a critical role in maintenance of luteal function in the ewe and cow. Moor & Rowson (1966a,b) demonstrated that the ovine conceptus must be present in the uterus by approximately Day 12 after estrus if luteal maintenance is to occur. Subsequently, the conceptus signal was identified as being proteinaceous in nature, and its intrauterine infusion caused cycle extension in sheep. The signal from the ovine conceptus is oTP-1 and has been the object of extensive study. Purified oTP-1 extended interestrous intervals and recently has been shown to be the only conceptus protein in oCSP responsible for extension of CL function during early pregnancy (Vallet et al., 1988).

The cow appears very similar to the sheep with respect to the mechanism for extension of CL function and the type of molecule used as the signal. Results of Northey & French (1980) and Betteridge et al. (1984) demonstrated that the bovine conceptus must be present in the uterus by approximately day 16 after estrus for extension of luteal function or for pregnancy to be established. As in the ewe, conceptus secretory proteins of the cow were reported to cause extension of luteal function. Proteins from ovine and bovine
conceptuses appeared to have antiluteolytic properties in that they altered PG dynamics of the uterus (see Thatcher et al., 1986a).

Of particular interest was the finding that the embryonic signal for extension of luteal function in these species appeared very similar. Heyman et al. (1984) demonstrated that trophoblastic tissues of the ovine and bovine conceptus extended luteal function in each species. Subsequently, Martal et al. (1984) reported that interspecies transfer of ovine and bovine trophoblastic tissues caused cycle extension in approximately 20% of the transfers. These results yielded the first true evidence that the mechanisms for luteal maintenance are similar in these species and may reflect evolutionary conservation of this process as it pertains to the establishment of pregnancy. Concurrent to the research by Martal et al. (1984), Knickerbocker et al. (1986b) demonstrated that bCSP extended interestrous intervals.

Studies were then carried out to determine if some component of bCSP is homologous to oTP-1, the conceptus signal responsible for extending CL function in sheep. Antibodies to oTP-1 previously developed (Godkin et al., 1982) were utilized in an Ouchterlony double diffusion system to determine if some component of bCSP cross-reacted with anti-oTP-1 antibody (chapter 2). Components of bCSP did cross-react with anti-oTP-1 antibodies and precipitin patterns demonstrated at least partial homology between components in oCSP and bCSP, implying conservation of antigenically similar sequences in the proteins. Immunoprecipitation analysis confirmed these results in that components of both bCSP and oCSP were immunoprecipitated with
anti-oTP-l antibody. The ovine conceptus signal, oTP-l, consists of one molecular weight species (19 kDa) that migrates as an acidic group with 3-4 isoelectric variants by 2-D SDS-PAGE (pI 5.3-5.7). Analysis of oTP-l has also shown that it does not contain any N-linked carbohydrate moieties (Anthony et al., 1988).

In contrast, anti-oTP-l antiserum immunoprecipitated seven isoelectric variants from bCSPs (pI 6.5-6.7), three of which migrated at 22 kDa, another three at 24 kDa and one species at 26 kDa, so some heterogeneity exists between the putative conceptus signals for these two species. The bovine conceptus proteins immunoprecipitable with antibodies to oTP-l have been called bTP-l.

Although oTP-l and bTP-l are both immunoprecipitable with anti-oTP-l antibody, the components of bTP-l are slightly more basic and of higher molecular weight than oTP-l. This raised the question as to why these immunologically related molecules of the ewe and cow possessed such different physical characteristics. It had already been demonstrated that oTP-l is not glycosylated, so it was determined whether glycosylation of bTP-l could account for differences in size and charge of the proteins. Incubation of bovine conceptuses with tunicamycin, an inhibitor of N-linked glycosylation, resulted in secretion of bTP-l components which were 4 to 6 kDa smaller (eg. 18 kDa) than the naturally secreted form of bTP-l (chapter 3). Immunoreactive components of bCSP from tunicamycin-treated conceptuses also migrated as a single band. Subsequent results from experiments utilizing deoxymannojirimycin, endoglycosidase-H and concanavalin-A Sepharose affinity
chromatography demonstrated that the 22 and 24 kDa forms of bTP-1 are differentially processed (Fig. 6-1). The 22 kDa form of bTP-1 is high-mannose and the 24 kDa form is a complex-type glycoprotein. Two possible mechanisms exist to explain differential glycosylation. The bTP-1 complex may arise from a single gene, which results in release of some high-mannose bTP-1, while some of the bTP-1 is further processed in the golgi apparatus to form complex-type bTP-1. Alternatively, bTP-1 complex glycoproteins might arise from multiple gene products which may result in insertion or deletion of single amino acids. If this were the case, the different gene products might be targeted for degree of post-translational glycosylation as a result of these modifications in their primary structure. This would represent a mechanism whereby the cell determines whether post-translational processing results in formation of high-mannose or complex-type bTP-1.

It is particularly interesting to note that the non-glycosylated form of bTP-1 (18 kDa) is very close in size to the secreted form of oTP-1 (19 kDa). This indicates that glycosylation represents evolutionary divergence of the conceptus signals secreted by these species. The importance of the glycosylation of bTP-1 has yet to be discovered. Presence of sugar units might be necessary for binding to receptors or to make bTP-1 less sensitive to proteolytic degradation in the uterus. Alternatively, glycosylation of bTP-1 may serve no specific role: note that ovine trophoblastic vesicles, which secrete a non-glycosylated oTP-1 signal, can extend estrous cycles in cattle to a limited extent (Martal et al., 1984).
Fig. 6-1. Proposed model for differential glycosylation of the components of the bTP-1 complex. Cotranslational processing results in addition of the core carbohydrate structure to bTP-1 as it is synthesized by the ribosomes. This common precursor of bTP-1 components is then transported to the golgi apparatus where it is modified to the high-mannose type bTP-1. There are two possible fates of this form of bTP-1. Some is released from the golgi, packaged into secretory vesicles and secreted as the high-mannose form. Other molecules are further processed in the golgi by addition and deletion of various sugar moieties to generate the complex-type glycoprotein form of bTP-1. In this fashion, one translation product is converted into two molecular-weight forms of bTP-1. In addition, isoelectric variants of bTP-1 exist and they likely represent several gene products of post-transcriptional processing of mRNA.
A combination of saturated ammonium sulfate precipitation, CM-Sepharose and DEAE ion exchange chromatography and HPLC gel filtration chromatography was utilized to purify bTP-1 (chapter 4). Purity of the preparation was confirmed by 2-D SDS-PAGE. The array of proteins detected by this procedure closely resembles that of bTP-1 detected by immunoprecipitation and fluoroigraphy (chapter 2), with one exception. Rather than seven isoelectric species as detected in chapter 2, about 10 isoelectric variants were detected by silver stain and all of these cross-reacted with anti-oTP-1 antibody, as shown utilizing immunoblot procedures (chapter 4). These additional isoelectric species likely are less abundant variants that are detectable due to enrichment of the bTP-1 complex during purification. Alternatively, in this experiment, purified bTP-1 which was derived from three consecutive 24 h culture periods of individual conceptuses, may in fact be more heterogeneous than bTP-1 derived exclusively from the first 24 h of culture.

The purified bTP-1 complex of proteins was utilized to determine if it represented the bovine conceptus "signal" responsible for initiating the events which result in extended CL function. In the first experiment, BSA, bCSP or bTP-1 were infused into the uterine lumen of cattle. Treatment with bTP-1 resulted in extension of luteal function and interestrous intervals. Treatment with bTP-1 complex also resulted in an attenuation of PGF release by the uterus. Vena cava plasma concentrations of PGF for bTP-1-treated cows tended to be lower than that of BSA-treated controls and were much less variable. This indicated that bTP-1 reduced uterine secretion of PGF
and attenuated lytic pulsatile release. These data agreed with those of Knickerbocker et al. (1986b) who reported that bCSP significantly attenuated PGF secretion by the uterus.

An additional goal was to determine the mechanism of action whereby bTP-1 attenuates uterine PGF secretion. Basu & Kindahl (1987) and Gross et al. (1988a) demonstrated the presence of an endometrial inhibitor of PG enzymes during the bovine estrous cycle and found its activity to be increased during early pregnancy. It was hypothesized that bTP-1 might mediate its anti-luteolytic role by inducing endometrial synthesis of inhibitors to PG synthesizing enzymes (Thatcher et al., 1986a). Gross et al. (1988a) demonstrated that bCSP attenuated PGF secretion and increased PG inhibitor activity in bovine endometrial explant cultures. With these results in mind, an experiment was designed to examine effects of bTP-1 on endometrial function (chapter 5).

Treatment with bTP-1 and bCSP attenuated PGF secretion by endometrial explants (chapter 5). This provided further evidence that bTP-1 is the conceptus derived anti-luteolytic agent of early pregnancy. These results again are in agreement with those of Gross et al. (1988a) who reported that bCSP attenuated PGF secretion by endometrial explants. Induction of an endogenous intracellular endometrial prostaglandin synthesis inhibitor was demonstrated for endometrial explants incubated with either bCSP or bTP-1. These results are in agreement with those of Gross et al. (1988a,c) and indicate that the conceptus, anti-luteolytic mechanism is mediated by induction of PG synthesis inhibitors.
Interestingly, bCSP and bTP-1 had differential and significant effects on PGE-2 secretion by endometrial explants (chapter 5). While bTP-1 tended to increase PGE-2 secretion, bCSP tended to decrease PGE-2 secretion by endometrium and the PGE-2 response was significantly different for bTP-1 and bCSP treatment. This indicated that some component of bCSP, but not bTP-1 may attenuate PGE-2 secretion. The effect of bTP-1 alone is to increase PGE-2 secretion which may represent uterine metabolism of PGF-2\alpha to PGE-2 or redirection of PG synthesis from PGF-2\alpha to PGE-2.

Culture of endometrial explants with bTP-1 or bCSP decreased incorporation of radiolabel into secreted and tissue proteins. These results are in agreement with Bartol et al. (1981) who found total recoverable protein in uterine flushings of pregnant cattle to be lower in pregnant cattle prior to day 19 when compared to cyclic flushings. More recently, Gross et al. (1988a) reported that bCSP attenuated incorporation of radiolabel into both secreted and tissue proteins. The exact role that shifts in endometrial production of proteins play is not known but likely represents changes to enhance survival of the conceptus.

Based upon data contained in this dissertation, a mechanism for the bovine antiluteolytic pathway during early pregnancy can be proposed (Fig. 6-2). During the estrous cycle, follicular estrogens induce synthesis of oxytocin receptors, which upon activation by ovarian and or pituitary derived oxytocin, stimulates PGF-2\alpha synthesis and secretion by the uterus (McCracken et al., 1984). This luteolytic hormone initiates regression of the CL, reduction of P4
Fig. 6-2. Proposed mechanism for the bovine antiluteolytic pathway during early pregnancy.
concentrations to low levels and a return to estrus. As proposed in this dissertation, the conceptus blocks this cascade of events through the secretion of bTP-1. This conceptus hormone likely binds to the epithelium of the endometrium to induce synthesis of an intracellular endometrial inhibitor of prostaglandin synthesizing enzymes. This in turn leads to attenuated secretion of uterine PGF-2 in response to oxytocin allowing maintenance of CL function. This model forms a conceptual basis for devising means to manipulate this system to improve embryonic survival and also may provide a framework for studying mechanisms for maintenance of CL function in other species in which attenuated release of the uterine luteolysin is the mechanism for maintenance of the CL.
REFERENCES


Curl, J.S., Thatcher, W.W. & Bartol, F.F. (1983) In vitro production of PGF\(_2\alpha\), PGF\(_{2\alpha}\) and 13,14-dihydro-15-keto-PGF\(_{2\alpha}\) (PGFM) by endometrial tissue from cattle at day 17 of pregnancy or the estrous cycle. J. Anim. Sci. 57(Suppl. 1), 327, Abstr.


mannosidase inhibitor blocking conversion of high mannose to

by blastocyst and early embryonic tissue of various species. J.
Reprod. Fert. 60,409-417.

Geisert, R.D., Renegar, R.H., Thatcher, W.W., Roberts, R.M. & Bazer,
Interrelationships between preimplantation development of the pig
27,925-939.

Geisert, R.D., Zavy, M.T., Biggers, B.G., Garret, J.E. & Wettemann,
early conceptus expansion in the bovine. Anim. Reprod. Sci. 16,
11-25.

by prostaglandin E2 during the estrous cycle in the cow. A
preliminary report. Theriogenology 19,693-700.

Ginther, O.J. (1968) Influence of exogenous progesterone and the
uterus on ovarian activity in sheep. Endocrinology 83,613-615.

Ginther, O.J. (1969) Length of estrous cycle and size of corpus
luteum in guinea pigs and sheep treated with progesterone at
different days of the estrous cycle. Amer.J. Vet. Res.

Ginther, O.J. (1974) Internal regulation of physiological processes
39,550-564.

Ginther, O.J. (1981) Local versus systemic uteroovarian relationships

Casida, L.E. (1967) Effects of oxytocin administration on the
estrous cycle of unilaterally hysterectomized heifers. J.

of the uterus and ovaries and the unilateral luteolytic effect of
the uterus: a local venoarterial pathway between uterus and ovary


BIографICAL SKETCH


The author then continued his education at Cornell University in Ithaca, NY, graduating with a Bachelor of Science degree in Animal Science.

The author entered North Carolina State University in August 1981 in pursuit of a Master of Science degree under the direction of Dr. Jack H. Britt. In August 1984 he received his Master of Science degree in animal science. His master's thesis was titled "Influence of Altered Corpus Luteum Function on Reproductive Hormones and Reproductive Performance of Dairy Cattle."

In May 1984, he began his doctoral program under the direction of Dr. William W. Thatcher in the Department of Dairy Science at the University of Florida in Gainesville. Upon completion of his Doctor of Philosophy degree the author will continue his work in reproductive physiology at the USDA, ARS Forage and Livestock Research Laboratory in El Reno, OK, under the direction of Dr.
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 W. Thatcher, Chair
Professor of Dairy 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.

Fuller W. Bazer
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.

Peter J. Hansen
Assistant Professor of Dairy 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. Buhl
Assistant Professor of Biochemistry and Molecular Biology

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

August, 1988

Dean, College of Agriculture

Dean, Graduate School