This is to certify that the

dissertation entitled

INFLUENCE OF ENERGY BALANCE ON CONCENTRATIONS OF LH RECEPTORS AND CHOLESTEROL IN BOVINE LUTEAL CELLS.

presented by

Trudy Lynn Hughes

has been accepted towards fulfillment
of the requirements for
Doctor of Philosophy degree in Animal Science

Ralph L. Fogwell
Major professor
Ralph L. Fogwell

Date 11/3/92

INFLUENCE OF ENERGY BALANCE ON CONCENTRATIONS OF LH RECEPTORS AND CHOLESTEROL IN BOVINE LUTEAL CELLS

By

Trudy Lynn Hughes

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1992

ABSTRACT

INFLUENCE OF ENERGY BALANCE ON CONCENTRATIONS
OF LH RECEPTORS AND CHOLESTEROL
IN BOVINE LUTEAL CELLS

By

Trudy Lynn Hughes

Energy balance can affect function of bovine corpora lutea (CL). My objective was to determine the effect of energy balance on concentration of receptors for luteinizing hormone (LH) and metabolism of cholesterol in bovine CL. Holstein heifers were fed diets to gain (Control, n=7) or maintain (Restricted, n=7) body weight. On day 12 after the second synchronized estrus, CL were removed. Diet did not affect serum concentration of progesterone, basal or LH-stimulated production of progesterone *in vitro*, or initial concentration of cholesterol in CL. Loss of body weight was associated positively with increased concentration of esterified cholesterol in CL. Compared with Controls, CL weighed less and concentration of LH receptors tended to be greater in luteal cells of Restricted heifers. Based on the change in cholesterol concentration during incubation, *de novo* synthesis of cholesterol was highest in CL of Control heifers. Based on correlation analysis, luteal cells of Controls used newly synthesized cholesterol for steroidogenesis. In

contrast, luteal cells of Restricted heifers appeared to use esterified cholesterol for steroidogenesis.

Objectives of Experiment II were to determine the effect of negative energy balance on: 1) activity of luteal HMG-CoA reductase, 2) incorporation of ^{3}H-cholesterol into progesterone by CL, and 3) the effect of concentration of acetate, glucose or ketones on luteal steroidogenesis. The experimental design was similar to Experiment I except Restricted animals lost weight. Compared with Controls, concentration of progesterone in serum tended to be reduced and CL weighed 49% less in Restricted heifers. Diet did not affect luteal production of progesterone *in vitro*, or activity of HMG-CoA reductase in CL. However, *de novo* synthesis of cholesterol appeared to be higher in CL of Controls. Concentration of receptors for LH and esterified cholesterol increased, and incorporation of ^{3}H-cholesterol into progesterone decreased in luteal cells of Restricted animals. Acetate and glucose did not consistently affect steroidogenesis. Ketones increased luteal production of progesterone only in Controls.

The most dramatic effects of dietary energy restriction on bovine CL were: reduced luteal weight, increased concentration of receptors for LH, and increased concentration of esterified cholesterol.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support of the Department of Animal Science. I thank my major professor, Dr. Roy Fogwell, for his dilligent assistance and advice. I also thank the other members of my graduate committee: Drs. Dale Romsos, H. Allen Tucker, and William Wells for their time and guidance throughout my graduate program.

Several members of the Animal Reproduction Laboratory contributed their time and support for this research. In particular, thanks are due Debbie Peters, Geoff Dahl and Larry Chapin for their invaluable assistance. My thanks to Dr. Ulrey, Dr. Pao, Dr. Allen and Jeff Bieszki and the Millipore Corporation for loans of equipment and/or expertise which were necessary for analyses. In addition, I am very appreciative of the cheerful assistance of Marilyn Emery, Joanna Gruber and Faye Cotton. I am grateful to Jim Liesman for his support and advice on a variety of technical questions as well as his friendship.

Finally, I wish to thank Jeff Bieszki for his continuous encouragement and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

ABBREVIATIONS

4-APP	4-aminopyrazolopyrimidine
ACAT	acyl coenzyme A transferase
ACTH	adrenocorticotrophic hormone
BSA	bovine serum albumin
BW	body weight
cAMP	adenosine 3′,5′-cyclic monophosphate
CE	esterified cholesterol
CEH	cholesterol ester hydrolase
CL	corpus luteum, corpora lutea
CO_2	carbon dioxide
cpm	counts per minute
cpm_{crt}	counts per minute corrected for maximum binding
C_{scc}	cholesterol side chain cleavage
dbcAMP	dibutyryl cAMP
fMol	femtamole(s)
FSH	follicle stimulating hormone
g	gravity
gm	gram(s)
h	hour(s)
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotropin
HDL	high density lipoprotein(s)

HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HMG-CoA reductase	hydroxymethlyglutaryl coenzyme A reductase
HPLC	high performance liquid chromatography
IU	International Units
iv	intravenous(-ly)
K_a	affinity constant or equilibrium association constant
kg	kilogram(s)
LDL	low density lipoproteins
LH	luteinizing hormone
LPL	lipoprotein lipase
LPDS	lipoprotein deficient serum
Mcal	megacalorie(s)
MEM	minimum essential media
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
mMol	millimole(s)
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NaF	sodium fluoride
ng	nanogram(s)
nm	nanometer(s)
nMol	nanomole(s)

O_2	oxygen
$P\text{-}450_{scc}$	side chain cleavage cytochrome P-450
PBS	phosphate buffered saline
pg	picogram(s)
PG	prostaglandin $F_{2\alpha}$
PMSG	pregnant mare serum gonadotropin
pp-1	protein phosphatase-1
ppm	parts per million
SCP2	sterol carrier protein 2
sec	second(s)
SE	standard error
SED	standard error of the difference
SEM	standard error of the mean
µg	microgram(s)
µMol	micromole(s)
VLDL	very low density lipoprotein(s)
vol	volume

INTRODUCTION

The CL is the primary source of progesterone in the serum of non-pregnant cattle. Concentration of progesterone in serum must be of sufficient magnitude during the estrous cycle preceding insemination for a successful conception to occur (Folman et al,. 1973; Corah et al., 1974; Carstairs et al., 1980; Kimura et al., 1987; Mee et al., 1987). In beef or dairy heifers with normal luteal function, treatments which increased the concentration of progesterone in serum after insemination do not affect pregnancy rates (Sreenan and Diskin, 1983; Garret et al., 1987; Stewart et al., 1987). However, in ewes or cows with subnormal luteal function, treatments which increased the concentration of progesterone in serum after insemination improved pregnancy rates (Kittok et al., 1983; Sreenan and Diskin, 1983; Thatcher et al., 1987). Thus, concentration of progesterone in serum immediately following insemination also affects pregnancy rate. Finally, presence of a CL, or an exogenous source of progesterone, is necessary in the first 200 days of gestation to maintain pregnancy (Johnson and Erb, 1962; Estergreen et al., 1967; Chew et al., 1979; Pimental et al. 1986).

In the majority of dairy cows a period of negative energy balance occurs during the first third of lactation. Negative

energy balance occurs primarily because energy intake does not fully supply the energy required for lactation (Bauman and Currie, 1980; Youdan and King, 1977; Villa-Godoy et al., 1988). In lactating dairy cows, negative energy balance postpartum is associated with reduced luteal function (Villa-Godoy et al., 1988). As previously described, luteal function which is below normal levels can reduce conception and pregnancy rates in cattle. Since the majority of dairy cattle experience negative energy balance during the first third of lactation and during this time dairy managers desire cattle to conceive, the relationship between negative energy balance and reduced luteal function in cattle is economically important.

There are conflicting results between reports regarding the relationship between milk production and reproductive performance and reports regarding the relationship between energy balance and reproductive performance (Hillers et al., 1984; Villa-Godoy et al., 1988; Butler and Smith, 1989). The conflicts may in part be due to the different measures of reproductive performance used and that yield of milk is not always highly predictive of energy balance. But in general, as more body condition is lost or more milk is produced, there are increased days to first service and decreased first service conception rates (Faust et al., 1988; Butler and Smith, 1989). Indeed, as milk production by dairy cows increased 1500 kg (33%) between 1951 and 1973, first service conception rate declined from 66% to 50% (Butler and Smith, 1989). Thus, negative energy balance may interfere with the

dairy industry's goal of a 12 to 13 month calving interval.

To investigate the relationship between negative energy balance and luteal function numerous studies have used heifers fed diets of different energy content as a model (Hill et al., 1970; Gombe and Hansel, 1973; Apgar et al., 1975; Spitzer et al., 1978; Imakawa et al., 1983; Harrison and Randel, 1986; Villa-Godoy et al., 1990). Diet did not affect concentration of progesterone in luteal tissue (Apgar et al., 1975; Imakawa et al., 1983; Harrison and Randel, 1986). However, restricted energy intake was associated with decreased weight of CL in all but one of these reports (Imakawa et al., 1983). Restricted energy intake was also associated with decreased concentration of progesterone in serum in some (Hill et al., 1970; Gombe and Hansel, 1973; Apgar et al., 1975; Imakawa et al., 1983; Villa-Godoy et al., 1990) but not all (Spitzer et al., 1978; Harrison and Randel, 1986) studies. Variation in results among these reports is apparently due to the difference in degree of energy balance achieved between treatment groups within a study. Imakawa et al. (1983) demonstrated that the concentration of progesterone in serum is highest in heifers gaining weight, intermediate for heifers maintaining body weight and lowest in heifers losing weight. In fact, concentration of progesterone in serum during the luteal phase of the estrous cycle is correlated positively with change in body weight of heifers (Imakawa et al., 1986b).

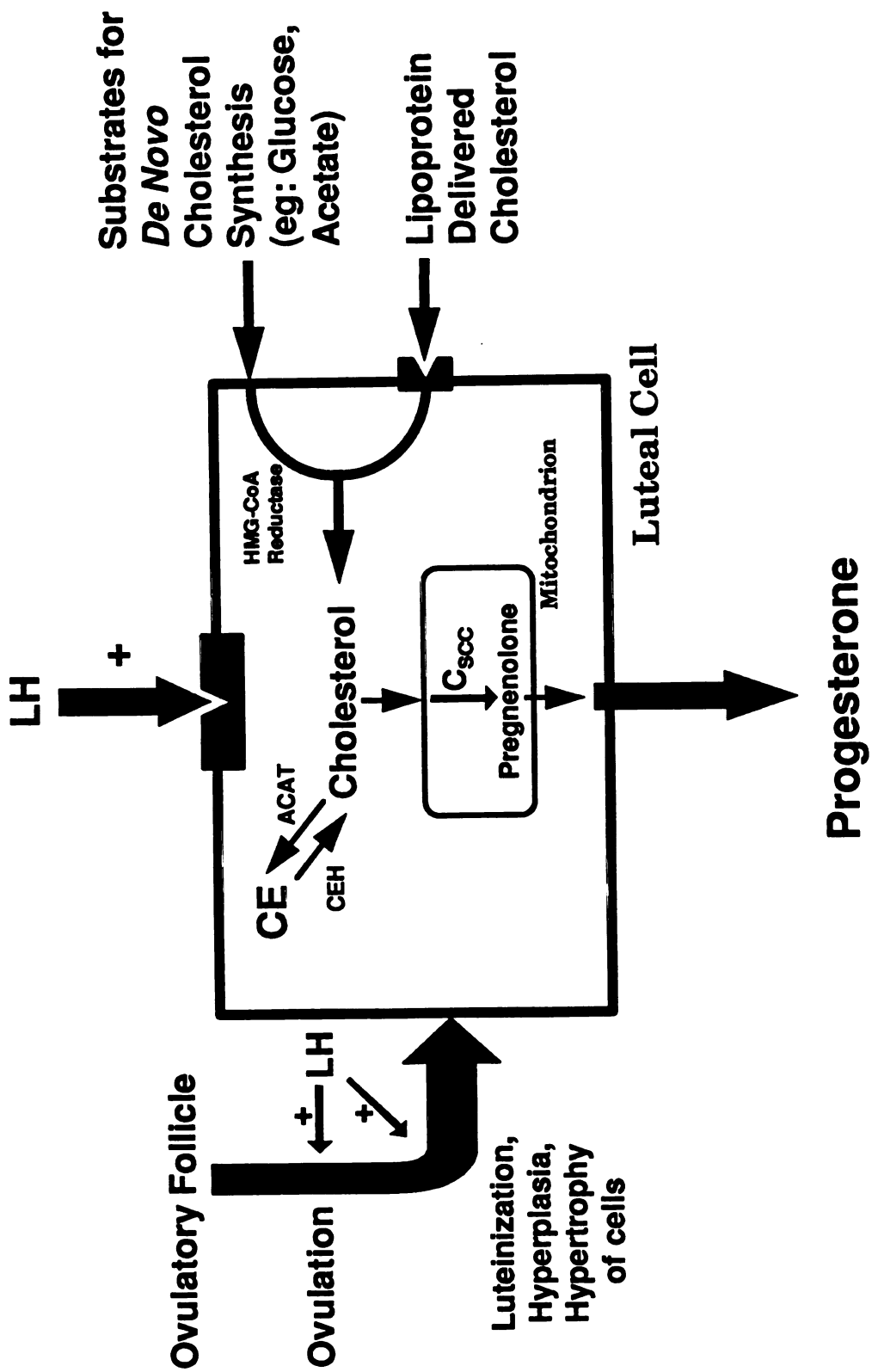
Negative energy balance reduced basal production of

progesterone by luteal cells of heifers in moderate body condition (Villa-Godoy et al., 1990). In addition, reduced energy intake decreased the ability of luteal tissue to synthesize progesterone in response to LH *in vitro* (Apgar et al., 1975; Imakawa et al., 1983; Villa-Godoy et al., 1990). Thus, reduced concentration of progesterone in serum of animals in negative energy balance may result from reduced weight of CL combined with reduced function of luteal cells. However, the mechanisms by which energy balance affects weight or function of CL are not well understood. It is of interest to understand the mechanisms by which energy balance alters luteal function since reduced concentration of progesterone in serum negatively affects reproductive performance. Therefore, the overall objective of this research was to investigate several mechanisms by which energy balance may alter luteal function.

A review of normal luteal development and function is presented to demonstrate aspects of luteal function which may be affected by energy balance and to establish the rationale for specific research objectives addressed in this dissertation. A model of development and function of bovine luteal tissue is included as a guide to the review (Figure 1). Information discussed is primarily from research conducted with cattle. However, when information from cattle was absent or limited, information from other species was included.

Figure 1. Control points in development and function of bovine luteal tissue. Factors which influence production of progesterone from a luteal cell are depicted. Luteinizing hormone (LH) stimulates luteinization, hyperplasia, and hypertrophy of cells from the ovulatory follicle. The number and type of luteal cells which develop during luteinization can affect subsequent luteal function. In addition, LH stimulates *de novo* production of cholesterol, uptake of serum lipoproteins, hydrolysis of esterified cholesterol and conversion of cholesterol into progesterone in luteal cells. Abbreviations include: acyl coenzyme A transferase (ACAT), cholesterol esterified to fatty acids (CE), cholesterol ester hydrolase (CEH), cholesterol side chain cleavage enzyme system (C_{scc}), hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase; the enzyme that catalyzes the rate limiting step in production of cholesterol).

Figure 1.


Substrates for
De Novo
Cholesterol
Synthesis
(eg: Glucose,
Acetate)
Lipoprotein
Delivered
Cholesterol
LH
+
HMG-CoA
Reductase
Luteal Cell
Mitochondrion
Cholesterol
ACAT
CE
CEH
C_{SCC}
Pregnenolone
Progesterone
Ovulatory Follicle
LH
+
+
Ovulation
Luteinization,
Hyperplasia,
Hypertrophy
of cells

REVIEW OF THE LITERATURE

Luteinizing Hormone is the Primary Luteotropin in Cattle

Luteinizing hormone is the primary luteotropin in cattle. LH is necessary for development of CL, maintenance of luteal cell morphology, and production of progesterone (Gospodarowicz and Gospodarowicz, 1972,1975; Hansel et al., 1973; Hoffman et al., 1974; O'Shaughnessy and Wathes, 1985a; Fairchild and Pate, 1987; Poff et al., 1988).

Passive immunization of heifers against LH from day 2 to day 6 postestrous (day 0 = day of estrus) reduced luteal weight and reduced the number of large luteal cells observed histologically in CL removed on day 11 (Snook et al., 1969). Conversely, administration of LH to ewes from day 5 to day 10 of an estrous cycle increased the weight of CL and increased the ratio of large to small luteal cells in CL collected on day 10 (Farin et al., 1985). In addition, number of receptors for LH in luteal tissue are correlated positively with weight of CL in ewes and heifers (Diekman et al., 1978; Spicer et al., 1981). Thus, change in concentration of LH in serum or change in number of receptors for LH in luteal tissue may influence development of CL. Energy balance may affect the positive relationship between LH and development of CL. Effects of energy balance on concentration of LH in serum will

be described (Concentration of LH in Blood). The effect of energy balance on number of receptors for LH on luteal cells has not been determined.

Mechanism of LH Action

LH increases steroidogenic function of luteal tissue by; 1) increased uptake of cholesterol associated with serum lipoproteins, 2) increased *de novo* cholesterol synthesis, 3) increased hydrolysis of esterified cholesterol stored within luteal tissue, 4) increased transport of cholesterol from various cellular locations to the inner mitochondrial membrane for the cholesterol side chain cleavage enzyme system (C_{scc}), and 5) increased activity of C_{scc}. The mechanism by which LH exerts these effects is generally accepted to be a cascade of events that begins when LH binds to its receptor in the plasma membrane (Rao et al., 1983).

After binding to its receptor, LH activates membrane bound adenylate cyclase and stimulates intracellular production of adenosine 3',5'-cyclic phosphate (cAMP; Ling and Marsh, 1977; Williams et al., 1978; May and Schomberg, 1984; Budnik and Mukhopadhyay, 1987; Smith, 1986). Acting as a second messenger, cAMP activates cAMP dependent protein kinase (Menon, 1973; Azhar and Menon; 1975; Caron et al., 1975; Darbon et al., 1976; Ling and Marsh, 1977). As a result of LH-stimulated kinase activity, a variety of specific cell proteins are phosphorylated in luteal cells (Darbon et al., 1980). LH-stimulated phosphorylation increases the activity of enzymes in luteal cells such as cholesterol ester

hydrolase (CEH; Bisgaier et al., 1979) and side chain cleavage cytochrome P-450 ($P\text{-}450_{scc}$; Caron et al., 1975; Williams et al., 1978).

In addition to phosphorylation of specific proteins, LH increases the concentration of a variety of proteins in ovarian tissue (Landefeld et al., 1979; Mittre et al., 1990). Some proteins that increase after LH stimulation are involved in steroidogenesis. Specifically, LH increased the concentration of receptors for lipoproteins (Savion et al., 1981a; Hwang and Menon, 1983; Golos et al., 1986; Ghosh and Menon, 1987) and increased the concentration of enzymes in the steroidogenic cascade (Savion et al., 1982; Funkenstein et al., 1983, 1984; Goldring et al., 1987; Rodgers et al., 1987a).

Response of luteal cells to LH requires receptors for LH. Decreased number or affinity of receptors for LH on luteal cells could explain the reduced response to LH noted in luteal cells from animals in negative energy balance (Apgar et al., 1975; Imakawa et al., 1983; Villa-Godoy et al., 1990). Similarly, change in activity of adenylate cyclase, or change in any other of the intracellular systems affected by LH could change the luteal response to LH. However, it is not known if energy balance affects the number or affinity of receptors for LH, or if energy balance affects any of the intracellular processes which are affected by LH.

Concentration of LH in Blood

Concentration of LH in blood fluctuates in a pulsatile pattern. Frequency and amplitude of the pulses of LH varies with stage of the estrous cycle (Rahe et al., 1980; Walters et al., 1984). From day 4 postestrus to day 11 postestrus, frequency of pulses of LH detected in plasma declined over 50% (Rahe et al., 1980; Walters et al., 1984) while amplitude of pulses remained unchanged (Walters et al., 1984) or increased (Rahe et al., 1980). Concentration of progesterone in ovarian venous plasma also fluctuates in a pulsatile manner concomitant with pulses of LH. Over 93% of pulses of LH are followed within 10 min by a pulse of progesterone in plasma (Walters et al., 1984; Proknor et al., 1986). The close association between pulses of LH and progesterone reflects the stimulatory action of LH on secretion of progesterone.

Compared with controls, restricted energy intake increased (Gombe and Hansel, 1973), did not change (Hill et al., 1970; Spitzer et al., 1978; McCann and Hansel, 1986), or decreased (Apgar et al, 1975; Richards et al., 1989) mean concentration of LH in plasma in heifers. One explanation for differences among studies may be that the difference in energy intake between groups of heifers was greater in some studies than in others (Apgar et al., 1975). Also, restricted energy intake may not result in negative energy balance, particularly if animals are fed as a group. In addition, frequency of sampling blood in these studies was 12 h or less. Therefore, frequency of sampling was inadequate to detect differences in

the pulsatile pattern of LH between energy balance groups (McCann et al., 1986).

When blood sampling was frequent (12 to 20 min intervals), Imakawa et al.(1986b) found energy balance affects the concentration of LH in blood of cattle. However, the effect of energy balance on concentration of LH was dependent on the stage of the estrous cycle. During the luteal phase of the estrous cycle there was no consistent relationship between change in body weight and concentration of LH in serum (Imakawa et al., 1986b). Additionally, other researchers have found energy balance did not affect mean concentration or the pulsatile pattern of release of LH on day 11 postestrus in heifers (Harrison and Randel, 1986; Villa-Godoy et al., 1990). Thus, it appears that energy balance does not affect concentration of LH in serum during the period of maximal luteal function. In contrast, during the follicular phase of the estrous cycle there was a positive relationship between body weight change and both mean concentration of LH in serum and amplitude of pulses of LH (Imakawa et al., 1986b). Reduced concentration of LH during the periovulatory period in animals in negative energy balance may set the stage for diminished luteal development as described below (Smith et al., 1986).

Periovulatory Events and Luteal Development

Two or three days before estrus, the ovulatory follicle of a cow is the largest follicle on either ovary (Dufour et

al., 1972; Ginther et al., 1989). As luteinization of the ovulatory follicle occurs, the major end product of steroidogenesis within this follicle shifts from estradiol-17β to progesterone (Henderson and Moon 1979; Channing, 1980; Ireland and Roche, 1982; Murdoch and Dunn, 1982; Dielman and Blankenstein, 1985). Concurrent with this shift in steroidogenesis and following ovulation, receptors for follicle stimulating hormone (FSH) on granulosa cells are lost or reduced (Ireland and Roche, 1982), while number of receptors for LH on luteinized cells are increased (Diekman et al., 1978; Spicer et al., 1981; Roser and Evans, 1983; Garverick et al., 1985).

Soon after ovulation, thin walled capillaries from the theca interna penetrate the basement membrane into the layers of granulosa cells (Balboni, 1983). Between 24 to 48 h after ovulation the basement membrane disappears and the distinction between the cells of the infolded theca interna and granulosa layers is lost (Donaldson and Hansel, 1965). Extensive vascularization of the CL (Koos and LeMarie, 1983) and hypertrophy and hyperplasia of luteal cells continues until approximately day 9 when size of CL and concentration of progesterone in serum are maximal (Bartol et al., 1981; Spicer et al., 1981; Wise et al., 1982; Garverick et al., 1985; Ginther et al., 1989). During the luteal phase of the estrous cycle, concentration of progesterone in serum is correlated positively both with weight of CL (Diekman et al., 1978; Spicer et al., 1981; Garverick et al., 1985) and blood

flow to the CL (Niswender et al., 1976; Wise et al., 1982). Concurrent with the morphological development of the Cl after ovulation, the concentrations of receptors for LH (Spicer et al., 1981), adenylate cyclase (Garverick et al., 1985), hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase; Rodgers et al., 1987a), and P-450_{scc} (Rodgers et al., 1986b) increase several fold in luteal cells.

Since negative energy balance reduced weight of CL, negative energy balance must have interfered with development of CL. However, factors that control growth and development of bovine CL are not fully understood. Thus, the mechanisms by which negative energy balance reduced weight of CL can only be speculated. Vascular development of the CL is one factor affecting luteal growth and development which may be sensitive to energy balance.

Blood flow to the ovary bearing the CL increases ≥ three fold between ovulation and time of maximal luteal development in ewes and cows (Niswender et al., 1976; Wise et al., 1982). It is unclear whether increased blood flow to the ovary bearing the CL is a cause or effect of increased luteal function. However, the mean ovarian capillary blood flow in the ovulatory ovary was 65% greater in ewes which subsequently demonstrated normal luteal function compared with ewes which developed abnormal CL (Brown et al., 1988).

What controls vascular development of the CL? A variety of factors which are angiogenic are found in ovarian tissue (Koos and LeMarie, 1983; Gospodarowicz et al., 1985; Redmer

et al., 1988; Koos, 1989). There is speculation that histamine derived from mast cells promotes the vascularization of the newly developing CL. Indeed, histamine can induce angiogenesis, vasodilation and can increase vascular permeability (Tharp, 1989). In addition, the release of histamine from mast cells appears to be controlled by gonadotropins.

Concentration of mast cells increases in thecal cells as follicles grow in size (Nakamura et al., 1987). Mast cells in the ovary degranulate and concentration of histamine in the ovary rises coincident with the gonadotropic surge in hamsters (Krishna and Terranova, 1985). Perhaps because of increased histamine release, increased concentration of LH in serum at estrus is associated with increased vascularity of the ovulatory follicle in rats (Terranova and Byrd, 1986). Treatment of ewes prior to ovulation with diphenhydramine, an antihistamine, reduced thecal hyperemia, decreased weight of follicular fluid and depressed subsequent luteal function (Halterman and Murdoch, 1986). As a result of the positive relationship between LH and vascular development of the ovulatory follicle, the periovulatory concentration of LH may affect development and function of the CL. Concentration of LH in serum is decreased during the periovulatory period in animals during negative energy balance (Imakawa et al., 1986b). Decreased concentration of LH in serum may interfere with vascularization of the ovulatory follicle and development of the CL. It is not known if energy balance affects the

vascular development of the ovulatory follicle.

Small versus Large Luteal Cells

Energy balance may alter the type of steroidogenic cells which develop in the CL, and as a result, luteal function may be affected. Steroidogenic cells comprising the CL may be classified into two types based on morphology, and functional differences (Table 1). Anatomical features can be used to distinguish Type I (small) from Type II (large) luteal cells. In ovine and bovine CL, large luteal cells are characterized by: light chromatin pattern of large spherical nuclei; extensively folded plasma membrane; abundant large mitochondria with tubular cristae or small mitochondria with lamelliform or tubular cristae; lipid bodies present in peripheral areas of the cell; isolated stacks of rough endoplasmic reticulum; extensive smooth endoplasmic reticulum; and numerous membrane bound secretory granules. Small luteal cells (Type I) are characterized by: less infolding of the plasma membrane but with some microvillar projections; convoluted nuclei with dense chromatin and occasionally nucleoli; numerous lipid droplets; absence of secretory granules; abundant endoplasmic reticulum which is predominantly smooth; and mitochondria of uniform size with tubular cristae (Priedkalans and Weber, 1968a and b; Koos and Hansel, 1981; Rao et al., 1983; Farin et al., 1986). Small luteal cells originate from thecal cells of follicles, whereas large luteal cells originate from granulosa cells of follicles

Table 1. Characteristics of Steroidogenic Cells Within the Bovine CL

Characteristics	Type II Cells	Type I Cells	Reference
Cell diameter (μm)	38.4	17.2	O'Shea et al., 1989
Cell Volume (x $10^3 \mu m^3$)	29.6	2.7	O'Shea et al., 1989
Progesterone pg/cell/h	2.65	.14	Koos and Hansel, 1981
LH Stimulated Progesterone pg/cell/h	3.90	1.48	Koos and Hansel, 1981
LH Receptors per Cell (x 10^3)[a]	3.1	33.3	Fitz et al., 1982
Cell #/CL on Day 12 Postestrus (x 10^6)	51	392	O'Shea et al., 1989
Cytoplasmic Volume Density			Priedkalns and Weber, 1968a
(%) Mitochondria	19.6	11.8	
(%) Lipid Droplet	4.6	8.8	

[a]Data determined from ovine luteal cells.

(Alila and Hansel, 1984).

Type of tissue preparation and criteria used to classify cells affects the results of studies on the cellular composition of CL. For example, using dispersed ovine luteal cells classified based on size, Schwall et al. (1986) determined that the ratio of small to large luteal cells decreased during the estrous cycle. These results conflict with the trend reported in the morphometric analysis by Farin et al. (1986). Similarly, conflicts exist between studies using sliced CL and dispersed luteal cells in cattle. For example, morphometric analysis of day 12 bovine CL indicated that the ratio of small to large luteal cells was 7.6:1 (O'Shea et al., 1989) similar to the ratio of Type I to Type II luteal cells noted by Farin et al.(1986) in ovine CL. However, in preparations of dispersed luteal tissue with bovine luteal cells classified based on size, the ratio of small to large luteal cells was 24.4:1 (Rodgers et al., 1988).

Discrepancies between studies appear due to differences in type of tissue preparation and in type of cell classification. Dispersion of luteal tissue can cause selective loss of large luteal cells (O'Shea et al., 1989). In addition, there are several reports that tissue dispersion reduces viability of large cells compared to small cells (Ursely and Leymarie, 1979, Fitz et al., 1982; Hoyer et al., 1984; O'Shaughnessy and Wathes, 1985a). Classifying luteal cells based on size alone does not discriminate well between type of luteal cells. Farin et al. (1986) noted that diameter

of Type II cells on day 4 (21.5 μm) is similar to diameter of small cells. Consequently, Type II cells may be misclassified as small luteal cells on day 4. As a result of the poor ability to discriminate between Type I and Type II luteal cells on size alone, luteal cells classified only by their size may not accurately reflect the type of cells which are present. Because of the problems with tissue dispersion and discrimination between types of luteal cells based on their size, morphometric analysis of luteal cells in tissue slices gives the most reliable information regarding the cellular composition of the CL. Thus, in the discussion of development of luteal cells morphometric data of CL were used preferentially.

Composition of steroidogenic cells in the CL varies with the age of the CL. Using morphometric analysis of cell types in ovine CL, Farin et al. (1986) found the number of Type I (small) luteal cells increased three fold between day 4 and 12 postestrus. Meanwhile, size of Type I cells remained constant. In contrast to Type I cells, number of Type II (large) luteal cells did not change between day 4 and 12 postestrus. However, during the same period diameter of Type II cells increased by 50% (Farin et al., 1986). Morphometric analysis of bovine luteal tissue throughout the estrous cycle has not been conducted. However, Donaldson and Hansel (1965) conducted a histological study of bovine luteal slices. Though number of large and small luteal cells were not reported, mitotic activity was found from ovulation to day 4

postestrus in Type II cells and from ovulation to day 7 postestrus in Type I cells. Thus, it seems reasonable to expect that increased number of small luteal cells and increased size of large luteal cells noted in ovine CL during the estrous cycle also occurs in bovine CL during an estrous cycle.

Large luteal cells secrete more progesterone per cell than do small cells from cattle and sheep (Table 1). However, small cells are more responsive to stimulation by LH than are large cells (stimulation is expressed as the ratio between progesterone produced with LH versus basal secretion: Ursely and Leymarie, 1978; Koos and Hansel, 1981; Fitz et al., 1982; Hoyer et al, 1984; Rodgers et al., 1985). Thus, changes in the population of luteal cells may have important implications for function and regulation of CL. For example, as CL become older the ratio of small to large cells increases in CL (Farin et al., 1986). Meanwhile, basal secretion of progesterone declines and secretion of progesterone in response to stimulation by LH increases in bovine luteal cells (% increase over basal; Milvae and Hansel, 1983; Rodgers et al., 1988).

The effects of negative energy balance on number and size of steroidogenic cells present in CL have not been determined in tissue examined morphometrically. However, in dispersed luteal tissue, negative energy balance increased the ratio of small to large luteal cells compared with tissue from heifers in positive energy balance (Villa-Godoy et al., 1990). The

authors suggested negative energy balance either interfered with the early mitotic activity in granulosa-lutein cells or hypertrophy of cells during luteinization. Since, on a per cell basis, large cells produce more progesterone than do small cells but are less responsive to stimulation by LH, one would predict that negative energy balance would result in decreased basal production of progesterone and increased responsiveness to LH stimulation. Indeed, luteal cells from moderately conditioned animals in negative energy balance produced less progesterone than did cells from animals in positive energy balance (Villa-Godoy et al., 1990). However, LH did not stimulate production of progesterone in luteal cells from heifers in negative energy balance (Villa-Godoy et al., 1990). There are two possible reasons why energy balance affects the ability of LH to stimulate production of progesterone: 1) energy balance affects number of receptors for LH on luteal cells, or 2) energy balance changes the intracellular events involved in production of progesterone.

Cholesterol Metabolism: A Key Event in Steroidogenesis

Cholesterol is required in luteal cells to maintain the integrity of plasma membranes and to serve as the precursor for progesterone. Bovine CL on day 11 postestrus contain approximately 34.9 μMol of cholesterol (Hafs and Armstrong, 1968), yet secrete 4.8 μMol of progesterone per h into the ovarian vein (Wise et al., 1982). Thus, a source of cholesterol is necessary to maintain luteal function.

Cholesterol in ovarian cells is from low density (LDL) or high density lipoproteins (HDL) in blood, or from *de novo* synthesis in luteal cells. A premise of this thesis is that energy balance affects the availability and use of cholesterol in luteal tissue. Change in availability and use of cholesterol in luteal cells could alter the ability of luteal cells to produce progesterone.

Cholesterol Delivered to Tissues by Lipoproteins

It has been suggested that cholesterol delivered by lipoproteins in blood may supply approximately 80% of the cholesterol used for adrenal and ovarian steroidogenesis in a variety of animal species (reviewed by Strauss et al.. 1981); Gwynne and Strauss, 1982). There are four lines of evidence which support this suggestion. 1) Introduction of radiolabelled cholesterol *in vivo* produced steroids of high specific activity. 2) Reduction in concentration of cholesterol in blood by pharmacological intervention, increased *de novo* cholesterol synthesis by the adrenal gland and ovary but also reduced production of steroids by these tissues. 3) Rates of *de novo* cholesterol synthesis in ovarian tissue determined *in vitro* were not sufficient to meet steroid production rates calculated *in vivo*. 4) Presence of lipoproteins in tissue culture media stimulated steroid production.

Most of the results listed above were from studies of animals other than cattle. But, elevated concentration of cholesterol in serum increased the concentration of

progesterone in serum of cows and heifers (Talavera et al., 1985; Williams, 1989). In addition, presence of lipoproteins in media can increase production of progesterone in bovine luteal cells in tissue culture (see Lipoproteins and Steroidogenesis in Luteal Cells). Thus, while the actual contribution of lipoprotein derived cholesterol to production of progesterone by bovine CL is not known, a role for lipoprotein derived cholesterol in bovine luteal steroidogenesis is likely. The physiological status of animals can affect the concentration of lipoproteins in blood, and the metabolism of lipoproteins by tissue. Therefore, it is important to review the role of lipoproteins in steroidogenesis and the effect of energy balance on lipoproteins.

Lipoproteins in Blood of Ruminants

Ruminants normally receive diets comprised only of plant products. Thus cholesterol in blood of ruminants is derived from endogenous biosynthesis (Nestle et al., 1978). Synthesis of cholesterol in ruminants occurs primarily in the small intestine and secondarily in the liver (Noble, 1981; Grummer and Carrol, 1988; Stranberg and Tilves, 1988). Cholesterol is transported in blood in association with proteins (apolipoproteins) and other lipids. The complexes of proteins and lipids are called lipoproteins. Lipoprotein complexes can be differentiated by their weight, hydrated density, chemical composition and site of origin (Table 2).

The amount and type of lipid in a lipoprotein molecule

and the associated apolipoproteins determines the structure and physiological function of a lipoprotein (Puppione, 1978; Schaefer et al., 1978; Gwynne and Strauss, 1982). The type of apolipoprotein present varies among classes of lipoproteins. For example, LDL particles contain primarily ApoB apolipoprotein. In contrast, HDL particles do not have ApoB, but instead primarily contain ApoA-I and II, and ApoC-II. Different apolipoproteins have different functions. For example, there are receptors for ApoB on plasma membranes for binding LDL. Subsequent to binding of LDL to receptors lipids in LDL are transferred to cells. In contrast, ApoA-1 and ApoC-II activate enzyme systems involved in lipolysis and transfer of lipids between lipoproteins (lectin:cholesterol acyl transferase, ApoA-I; lipoprotein lipase, ApoC-II. Brown and Goldstein, 1986; Schaefer et al., 1978). As a result of functional differences between apoliporoteins, the type of apolipopoproteins present affects the use of the lipoprotein by tissue. Thus, the total concentration of a particular lipid in blood is not highly informative. For example, over 75% of the cholesterol in plasma is associated with the HDL fraction in cattle, whereas in humans, 75% of the cholesterol in plasma is associated with the LDL fraction. Composition of the major lipoprotein classes in humans and cattle is presented (Table 2) to demonstrate the variation among species in relative abundance and characteristics of lipoproteins in blood.

Table 2. Lipoprotein Composition in Blood of Humans and Cows

Lipoprotein Class	Size Range (nm)[a]	Species	Density[b] gm/ml	% protein by weight[c]	Lipids (%)[d] TG[e]	C	CE	PL	C[f] in Plasma (mg/100 ml)[f]
Chylomicron	80 to 500	human	<1.006	2	87	1	3	9	<1
		bovine (ND)							
VLDL	30 to 80	human	.95 to 1.006	10	55	8	13	20	12
		bovine	<1.006	6	61	50	19	15	2
LDL	16 to 25	human	1.006 to 1.063	23	13	10	48	26	112
		bovine	1.006 to 1.040	19	37	13	27	23	5
HDL	7 to 13	human	1.063 to 1.210	55	9	4	33	53	25
HDL_1		bovine	1.040 to 1.063	ND	3	10	52	35	22
HDL_2		bovine	1.063 to 1.210	43.5	<1	6	52	44	71

[a]Size range in nanometers (nm) from human lipoprotein classes (Puppione, 1978).

[b]Density of human lipoproteins (Rawn, 1988), bovine lipoproteins (Raphael et al., 1973).

[c]Protein % by weight of human lipoproteins (Rawn, 1988), bovine lipoproteins (Kris-Etherton and Etherton, 1982).

[d]Lipid Composition by % of human lipoproteins (Rawn, 1988), bovine lipoproteins (Raphael et al., 1973).

[e]Abbreviations: Cholesterol, (C); triglycerides, (TG); cholesterol esters, (CE); phospholipids, (PL); very low density lipoprotein, (VLDL); low density lipoprotein, (LDL); high density lipoprotein, (HDL); not determined, (ND).

[f]Concentration of cholesterol in plasma (mg/dl) was determined from data of Raphael et al. (1973), Rawn (1988), and Chapman et al. (1980). Concentration of cholesterol in cholesterol esters was estimated by multiplying by 0.6.

Lipoproteins and Steroidogenesis in Luteal Cells

During a 2 h incubation, addition of bLDL or bHDL did not increase basal or LH induced synthesis of progesterone in bovine luteal tissue (Condon and Pate, 1981). Inhibiting cholesterol biosynthesis also did not alter synthesis of progesterone in bovine luteal tissue in a 2 h incubation (Armstrong et al., 1970). Thus, it appears bovine ovarian tissue depends on cholesterol stored within the tissue for steroidogenic substrate in short term incubations. In fact, free sterol concentration in homogenates of bovine CL decreased during a 1 h incubation, and 30 to 50% of the loss could be accounted for as progesterone synthesized (Hafs and Armstrong, 1968).

The duration of incubation of luteal cells affects the contribution of endogenous cholesterol to steroidogenesis. Specifically, cholesterol stored within bovine luteal cells is not sufficient to maintain steroidogenesis *in vitro* for more than a few hours. During incubations longer than 20 h, addition of whole calf serum to media increased progesterone secreted from bovine luteal cells two to three fold compared with luteal cells cultured without serum (Pate and Condon, 1982; Rodgers et al., 1987a). Cholesterol carried by lipoproteins is apparently the factor in serum that stimulates progesterone secretion. Addition of lipoprotein deficient serum (LPDS) produced 50% less progesterone than cells cultured with complete serum (O'Shaughnessy and Wathes, 1985b). In addition, bovine LDL (bLDL), bHDL or 25-OH

cholesterol added to media with LPDS restored basal production of progesterone to concentrations observed with whole serum (O'Shaughnessy and Wathes, 1985b; Rodgers et al., 1987a). The ability of LH or dbcAMP to stimulate secretion of progesterone is also highest in long term cultures containing either serum, bLDL or bHDL (Pate and Condon, 1982; O'Shaughnessy and Wathes, 1985b; Pate et al., 1987). Thus, lipoproteins contribute significantly to steroidogenesis when cells are cultured ≥ 20 h.

Both bLDL and bHDL caused concentration dependent increases in basal production of progesterone (O'Shaughnessy and Wathes, 1985b). Maximal production of progesterone achieved did not differ between cultures receiving bLDL or bHDL. However, more HDL (116 μg cholesterol/ml) than LDL (58 μg cholesterol/ml) was required to achieve the maximal production rate. Similarly, maximal dbcAMP stimulated production of progesterone was acheived with LDL at 232 μg cholesterol/ml and HDL at 174 μg cholesterol/ml. But, the ED_{50} for maximal dbcAMP-stimulated production of progesterone was 13 μg bLDL cholesterol/ml and 46 μg bHDL cholesterol/ml (O'Shaughnessy and Wathes, 1985b). Thus, bovine luteal cells are more sensitive to cholesterol transported in LDL than in HDL. However, cholesterol in blood of cattle is primarily associated with HDL (Raphael et al., 1973). As a result, the relative importance of cholesterol derived from LDL versus HDL is not clear. As described below, energy balance may affect the concentration of LDL in blood. In addition, energy

balance may affect the ability of luteal tissue to metabolize LDL. Thus, it is possible that energy balance may affect luteal function by altering the availability of cholesterol derived from LDL. The relationship between energy balance and LDL metabolism is reviewed.

Relationship between Cholesterol in Blood and Luteal Function

Compared with low fat diets normally fed to ruminants, diets high in fat (7 to 10% ether extract on a dry matter basis) increased the concentration of cholesterol in blood of ruminants over 70% (Nestle et al., 1978; Talavera et al., 1985; Williams et al., 1989). In particular, supplementary dietary fat preferentially increased LDL associated cholesterol in heifers and cows (Storry et al., 1980; Park et al., 1983). In addition to increasing the concentration of LDL in blood, a high fat diet increased the concentration of progesterone in serum during diestrus in Holstein heifers (Talavera et al., 1985) and improved the ability of exogenous gonadotropin releasing hormone to induce CL of normal duration and function in postpartum cattle (Williams, 1989). The positive association between concentration of LDL in serum and luteal function *in vivo* is consistent with the positive association between the concentration of LDL and function of luteal cells *in vitro*. Therefore, independent of energy balance, availability of LDL cholesterol may affect production of progesterone in cows.

The effect of energy balance on concentration of

cholesterol in blood has not been addressed directly. However, during a 4-day fast the concentration of HDL cholesterol increased while the concentration of LDL cholesterol decreased (Brumby et al., 1975). Thus, it appears that energy balance may differentially affect the concentration of LDL and HDL cholesterol in blood. Since bovine luteal tissue is most sensitive to LDL, reduced concentration of cholesterol associated with LDL in blood may diminish luteal function in cattle during negative energy balance.

Metabolism of Low Density Liporoteins in Ovarian Tissue

Catabolism of LDL occurs by receptor dependent and receptor independent pathways (Chait, 1983). Receptor mediated uptake of LDL accounted for at least 65% of LDL degraded by rat ovarian tissue (Carew et al., 1982). The receptor that binds LDL recognizes ApoB-100, the major apolipoprotein of LDL or VLDL in most species including cattle or ApoE present in VLDL and a subclass of HDL of human and rats (Schaefer et al., 1978; Eisenberg, 1984; Cordle et al., 1985; Bradley and Gianturco, 1986; Brown and Goldstein, 1986). However, ApoE is not a constituent of lipoproteins of cows (Cordle et al., 1985). Thus, the 'LDL' receptor binds only LDL in cattle.

Receptors with high affinity and specificity for LDL occur in a variety of bovine tissues (Kovanen et al., 1979). In cattle, the concentration of receptors for LDL in a tissue

is associated positively with the steroidogenic activity of that tissue. The highest concentration of LDL receptors are found in CL and adrenal glands of cattle (Kovanen et al., 1979). In addition, the concentration of messenger ribonucleic acid (mRNA) for LDL receptor was higher in active CL than in ovarian follicles and was undetectable in regressing bovine CL (Rodgers et al., 1987b).

After LDL binds to its receptor, the plasma membrane containing the LDL/receptor complex invaginates and is internalized to form endocytic vesicles. After endocytic vesicles fuse with cytoplasmic lysosomes, LDL is dissociated from receptors and receptors recycle to the cell surface or are degraded with LDL. Degradation of lipoproteins by lysosomal proteases and esterases releases unesterified cholesterol and amino acids into the cell (Gwynne and Strauss, 1982; Brown and Goldstein, 1986) and the concentration of free and esterified cholesterol increases in cells (Veldhuis et al., 1986; Aviram et al, 1988; Rinninger and Pittman, 1988). Following metabolism of LDL particles, increased intracellular concentration of cholesterol causes increased activity of acyl CoA transferase (ACAT), and reduced activity of HMG-CoA reductase (Kreiger et al., 1978; Chang and Limanek, 1980; Goldstein and Brown, 1984; Suckling and Stange, 1985; Brown and Goldstein, 1986; Aviram et al., 1988; Pate and Condon, 1989). It is interesting to note that the rate of degradation of LDL is positively correlated with the production of progesterone in luteinized porcine granulosa

cells (r=.88; Veldhuis and Gwynne, 1985a).

Hormones which increase steroidogenesis regulate the concentration of receptors for LDL in steroidogenic tissue (reviewed by Gwynne and Strauss, 1982). For example, ACTH and dbcAMP increased concentration of receptors for LDL, increased internalization and degradation of LDL and increased the concentration of esterified and unesterified cholesterol in bovine adrenal cells (Kovanen et al., 1979; Hotta and Baird, 1987). Similarly, human chorionic gonadotropin (hCG) and analogs of cAMP increased the production of progesterone and concentration of receptors for LDL in luteinized human and bovine granulosa cells, and CL of humans and rats (Carr et al., 1981; Savion et al., 1981a; Hwang and Menon, 1983; Soto et al., 1985; Golos et al., 1986).

In a negative feedback loop, concentration of receptors for LDL is also modulated by concentration of cholesterol in tissue. When the endogenous concentration of cholesterol was increased, LDL receptor synthesis decreased in luteinized porcine and human granulosa cells (Veldhuis et al., 1985b; Golos et al., 1986). When *de novo* cholesterol synthesis was blocked by compactin, hCG-stimulated LDL receptor synthesis increased in luteinized human granulosa (Golos et al., 1986). Addition of gonadotropins reduced the concentration of cholesterol in steroidogenic tissue (Flint et al., 1973; Balasubramaniam et al., 1977; Schuler et al., 1978; Ascoli and Freeman, 1985; Rajendran et al., 1985; Brody and Black, 1988). Reduced intracellular concentration of cholesterol may

in part explain the ability of gonadotropins to increase the concentration of receptors for LDL in tissue. However, gonadotropin-stimulated increase in LDL receptor synthesis is not entirely due to reduced cellular concentration of cholesterol. Human chorionic gonadotropin increased LDL receptor synthesis in luteinized human granulosa cells even when intracellular cholesterol stores were replenished by 25-OH cholesterol (Golos et al., 1986). Thus, intracellular concentration of cholesterol and gonadotropins have independent and interactive actions which modulate concentration of receptors for LDL.

In addition to gonadotropins such as hCG, metabolic hormones can influence the binding of LDL to steroidogenic tissue. Insulin and insulin-like growth factor I increased binding and degradation of LDL particles and increased production of progesterone by luteinized porcine granulosa cells (Veldhuis et al., 1986, 1987). There are no reports on the effect of energy balance on number of receptors for LDL in ovarian tissue. However, negative energy balance reduced the concentration of insulin (Villa-Godoy et al., 1990) and insulin like growth factor I (Brier et al., 1988; Rutter et al., 1989) in serum of cattle. Extrapolating from the positive effect of insulin and insulin like growth factor I on number of LDL receptors in porcine ovarian tissue, negative energy balance may reduce the concentration of receptors for LDL on bovine CL. If this reduction in receptors for LDL occurs, the tissue concentration of cholesterol might decrease

and impair steroidogenesis of luteal cells.

Esterified Cholesterol and Steroidogenesis

Cholesterol within steroidogenic tissue is esterified to fatty acids (esterified cholesterol) or is unesterified (free cholesterol; Strauss et al., 1981 for review). From 80 to 94% of the free cholesterol within fibroblasts, hepatocytes (Lange and Ramos, 1983) and luteinized granulosa cells (Lange et al., 1988) is associated with the plasma membrane, while the majority of esterified cholesterol is in lipid droplets within the cytoplasm (Strauss et al., 1981). Within tissue, there is interchange between the two pools of cholesterol. An enzyme in the endoplasmic reticulum, ACAT, esterifies free cholesterol to fatty acids, while cytosolic cholesterol ester hydrolase (CEH) hydrolyzes stored cholesterol esters to free cholesterol (Tuckey and Stevenson, 1980; Strauss et al., 1981; Suckling and Stange, 1985; Nichikawa et al., 1988). The percent of cholesterol in tissue which is esterified varies among species, tissues, and physiological states as described below.

The contribution of esterified cholesterol to steroidogenesis in bovine luteal tissue is not clear. In luteal tissue of cows only 10 to 23% of total cholesterol is esterified (Hafs and Armstrong, 1968). In addition, the concentration of free cholesterol decreased during a one hour incubation of bovine luteal tissue. Thirty to 50% of free cholesterol lost during the incubation was accounted for production of progesterone. Incubation did not affect the

concentration of cholesterol esters (Hafs and Armstrong, 1968). Thus, esterified cholesterol may not contribute to steroidogenesis in short term incubations. However, esterified cholesterol may contribute to bovine luteal steroidogenesis *in vivo*. Activity of CEH in bovine and ovine luteal tissue is stimulated by LH and cAMP (Bisgaier et al., 1979; Caffrey et al., 1979). In addition, between day 2 to 14 postestrus CEH activity increased in ovine luteal tissue and was correlated positively with concentration of progesterone in serum (Caffrey et al., 1979). Indeed, the concentration of cholesterol esters in bovine luteal tissue reached the nadir during maximal production of progesterone (Hafs and Armstrong, 1968).

Compared with CL collected from day 2 to day 11 postestrus, the concentration of cholesterol esters was higher in bovine CL collected from day 18 to 20 postestrus (Hafs and Armstrong, 1968). By day 18 postestrus, concentration of progesterone in serum and ability of luteal cells to secrete progesterone are reduced (Armstrong and Black, 1966; Spicer et al., 1981; Milvae and Hansel, 1983; Rodgers et al., 1988). Thus, the concentration of cholesterol esters in bovine luteal tissue is associated negatively with production of progesterone. What causes increased concentration of esterified cholesterol in luteal tissue during reduced luteal function? Activity of CEH affects ability of tissue to mobilize esterified cholesterol for steroidogenesis. However, activity of CEH in ovine luteal tissue actually continues to

increase during luteolysis (Caffrey et al., 1979). Thus, activity of CEH does not explain the increase in concentration of esterified cholesterol late in the estrous cycle. It seems most probable that accumulation of cholesterol esters during luteolysis is due to decreased concentration and activity of components of the C_{scc} enzyme system noted in regressing bovine CL (Rodgers et al., 1986b, 1987b). Conversion of cholesterol to pregnenolone by C_{scc} is the rate limiting step in steroid biosynthesis (Strauss et al., 1981 for review). Decreased activity of the C_{scc} system can result in a build up of free cholesterol in the cytosol. Increased concentration of free cholesterol stimulates esterification of cholesterol in luteal cells (Strauss et al., 1981). Thus, the concentration of cholesterol esters in CL may be an indicator of activity of the C_{scc} enzyme system in bovine luteal tissue. It is not known if energy balance affects the concentration of cholesterol esters in luteal tissue.

De Novo Cholesterol Synthesis

Importance of *De novo* Cholesterol Synthesis in Luteal Tissue

Cholesterol derived from serum lipoproteins appears to make a significant contribution to bovine luteal steroidogenesis. There are several pieces of evidence which lead me to suggest that *de novo* cholesterol synthesis may also be an significant contributor to production of progesterone by bovine CL. In addition, there are several factors in the *de novo* synthesis of cholesterol which may be affected by energy

balance. Thus, a discussion of *de novo* synthesis of cholesterol is important to this review of literature.

As mentioned earlier, bovine luteal cells depend on endogenous cholesterol for steroid production in short term cultures. However, when bovine cells are cultured longer than 20 h without exogenous cholesterol, *de novo* cholesterol synthesis contributes to the production of progesterone (Pate and Condon, 1989). O'Shaughnessy and Wathes (1985b) cultured bovine luteal cells with compactin and without lipoproteins for 48 h. Compactin blocked cholesterol synthesis and reduced basal and dbcAMP induced production of progesterone by 28% and 50% respectively. Thus, *de novo* synthesis of cholesterol contributed to production of progesterone by bovine luteal cells *in vitro*. Since lipoproteins in serum are available to CL in cattle, the contribution of *de novo* cholesterol synthesis to production of progesterone *in vivo* is not known.

Based on circumstantial evidence, it appears that *de novo* synthesis of cholesterol is important to bovine luteal steroidogenesis *in vivo*. For example, HMG-CoA reductase catalyzes the rate limiting step in synthesis of cholesterol. There is a positive relationship between the concentration of HMG-CoA reductase and production of progesterone by luteal tissue. Indeed, the concentration of HMG-CoA reductase in luteal tissue is highest during peak luteal function in cows (Rodgers et al., 1987a).

The proportion of total cholesterol which is esterified may also indicate how important endogenously synthesized

cholesterol is to steroidogenesis. A high proportion of esterified cholesterol in tissue is consistent with dependence on lipoprotein-derived cholesterol for steroidogenesis. Steroidogenesis in adrenal glands and ovaries of rats is dependent on cholesterol derived from high density serum lipoproteins (Anderson and Dietschy, 1978; Schuler et al., 1979, 1981; Swann and Bruce, 1986; Azhar et al., 1988). During maximal luteal function over 60% of cholesterol in luteal tissue of rats is esterified and the activity of HMG-CoA reductase is at a nadir (Schuler et al., 1979; Azhar et al., 1984, 1988). Normal human adrenal tissue utilizes primarily lipoprotein derived cholesterol for steroidogenesis (Gwynne and Strauss, 1982) and contains 14 fold more esterified than free cholesterol (Lehoux et al., 1984). In contrast to ovarian tissue, placental tissue of rats uses cholesterol primarily from *de novo* synthesis for steroidogenesis (Gibori et al., 1988). Unlike luteal tissue, cholesterol esters comprise less than 20% of the total cholesterol in placental tissue of rats (Gibori et al., 1988). Additionally, a cell line derived from a human adrenal carcinoma is dependent on *de novo* cholesterol synthesis for production of steroids and the concentration of free cholesterol was 4.5 fold greater than the concentration of cholesterol esters in these cells (Lehoux et al., 1984). Thus, usually when cholesterol in a tissue is predominantly non-esterified that tissue depends on *de novo* cholesterol synthesis for steroid precursor. Interestingly, approximately

80% of cholesterol is not esterified in bovine luteal tissue (Hafs and Armstrong, 1968).

The importance of *de novo* synthesis of cholesterol in steroid synthesis in the bovine CL is certainly unclear. But, based on *in vitro* studies and the circumstantial evidence outlined above, *de novo* cholesterol may have a role in steroid production in bovine CL. As described in the following sections, *de novo* synthesis of cholesterol offers several control points which may be affected by energy balance of an animal. Thus *de novo* cholesterol synthesis may be a mechanism by which energy balance can influence luteal function.

Activity of HMG-CoA Reductase

HMG-CoA reductase, the enzyme which catalyzes the rate limiting step of cholesterol biosynthesis, converts HMG-CoA to mevalonate by two successive NADPH dependent reductions. Activity of HMG-CoA reductase within cells is controlled by phosphorylation state of the enzyme, and concentration of the enzyme.

Phosphorylation reduces the activity of HMG-CoA reductase. Phosphorylation of HMG-CoA reductase is catalyzed by HMG-CoA reductase kinase. HMG-CoA reductase kinase kinase catalyzes phosphorylation of HMG-CoA reductase kinase. In contrast to HMG-CoA reductase, HMG-CoA reductase kinase is active when it is phosphorylated (Beg et al., 1980; Ingebritsen and Gibson, 1980).

Phosphatases within cells can dephosphorylate proteins, and as a result can inactivate HMG-CoA reductase kinase and

activate HMG-CoA reductase (Beg et al., 1980; Ingebritsen et al., 1983). Protein phosphatase-2c accounts for 60-70% of the dephosphorylating activity in homegenates of liver homogenates (Ingebritsen et al., 1983). However, HMG-CoA reductase is a transmembrane protein of the endoplasmic reticulum (Goldstein and Brown, 1984; Liscum et al., 1985). Protein phosphatase-1 (pp-1) is the only protein phosphatase which is associated with the microsomal fraction. Thus, Ingebrisen et al. (1983) and Cohen (1989) suggested pp-1 plays a major role in regulating the phosphorylation of HMG-CoA reductase *in vivo*. Indeed, Feingold et al. (1983) demonstrated phosphatase activity of microsomes from liver can increase activity of HMG-CoA reductase in liver over 2 fold.

Amount of HMG-CoA reductase which is unphosphorylated (active) varies between types of tissues and is affected by the energy balance of the animal. In intestinal cells of rats and humans approximately 80 to 85% of HMG-CoA reductase present is in the active form (Field et al., 1982; Gebhard et al., 1985), but in hepatocytes only 12 to 25% of HMG-CoA reductase is in the active form (Brown et al., 1979; Edwards et al., 1980; Kliensik et al., 1980; Angelin et al., 1984). The active form of HMG-CoA reductase accounts for only 20 to 40% of the enzyme present in luteal tissue of rats and rabbits ((Azhar et al., 1984, 1985, 1988; McLean and Miller, 1988).

State of phosphorylation appears to be one mechanism by which energy balance can rapidly affect the activity of HMG-CoA reductase and control the rate of *de novo* cholesterol

synthesis. Insulin and glucagon are believed to affect phosphorylation of HMG-CoA reductase in skeletal muscle, liver and adipose tissue by altering the activity of pp-1 (Cohen, 1989). For example, insulin reduced but glucagon increased the amount of HMG-CoA reductase which is phosphorylated in liver tissue of rats (Beg et al., 1980; Ingebritsen and Gibson, 1980; Zammit and Easom et al., 1987). Negative energy balance reduced the concentration of insulin in blood of cattle (de Boer et al., 1985; Harrison and Randel, 1986; Villa-Godoy et al., 1990). Decreased concentration of insulin could reduce the activity of pp-1. Decreased activity of pp-1 could subsequently reduce HMG-CoA reductase activity in luteal tissue. It is not known if insulin or glucagon control activity of HMG-CoA reductase in luteal cells. However, if the relationship between insulin and glucagon and activity of HMG-CoA reductase in luteal tissue is similar to the relationship in liver, negative energy balance would reduce *de novo* cholesterol synthesis in luteal tissue.

Concentration of HMG-CoA Reductase in Ovarian Tissue

Changes in metabolic status or changes in the concentration of insulin in blood can rapidly phosphorylate and thus decrease the activity of HMG-CoA reductase in hepatocytes (Zammit and Easom, 1987). However, long term (>2 h) regulation of HMG-CoA reductase activity in liver appears to be due primarily to change in total amount of enzyme present rather than change in the phosphorylation state of the enzyme (Brown et al., 1979; Kleinsek et al., 1980; Arebalo

et al., 1981; Popjak et al., 1985). Similar to the liver, the long term regulation of HMG-CoA reductase activity in ovaries appears to be through change in enzyme concentration.

Acute effects of gonadotropins on level of phosphorylation of HMG-CoA reductase in ovarian tissue have not been investigated. However, administration of hCG and LH increased HMG-CoA reductase activity within rat CL by 2 to 4 h (Schuler et al., 1981; Azhar et al., 1984). The increased activity noted at 4 hours was due to increased amount of enzyme present while ratio of active to inactive enzyme remained constant (Azhar et al., 1984). Similarly, changes in activity of HMG-CoA reductase in luteal tissue during pregnancy in rats or rabbits are due to changes in total enzyme present rather than changes in phosphorylation of the enzyme (Azhar et al., 1988; McLean and Miller, 1988).

The quantity of HMG-CoA reductase, rather than its level of activation, is also the most likely mechanism by which activity of HMG-CoA reductase is altered in bovine luteal tissue. Concentration of HMG-CoA reductase in bovine luteal tissue during the estrous cycle reflects the steroidogenic activity of the tissue. Peak concentration of HMG-CoA reductase is coincident with maximal luteal development and peak concentration of progesterone in serum (Spicer et al., 1981; Rodgers et al., 1987a). Conversion of radiolabelled acetate into cholesterol is a marker of activity of HMG-CoA reductase. Incorporation of acetate into sterols and cholesterol is highest in bovine CL which are actively

secreting progesterone (Armstrong and Black, 1966). Increased concentration of enzyme may be the mechanism by which LH stimulates HMG-CoA reductase activity and incorporation of acetate into cholesterol and progesterone in bovine luteal tissue (Savard et al., 1965; Armstrong and Black, 1968). Both dbcAMP and LH increased the synthesis and concentration of total HMG-CoA reductase in luteinized bovine granulosa cells and freshly isolated bovine luteal cells (Savion et al., 1982; Rodgers et al., 1987a). Inhibition of protein synthesis blocked the stimulatory effect of LH on the incorporation of acetate into progesterone (Savard et al., 1965). Increased synthesis of HMG-CoA reductase after dbcAMP-stimulation appears to be a response to reduced concentration of cholesterol within luteal cells following gonadotpic stimulation. When the use of cholesterol by P-450_{scc} was blocked, dbcAMP did not stimulate synthesis of HMG-CoA reductase (Rodgers et al., 1987a).

Food deprivation reduced the concentration of HMG-CoA reductase by 90% in hepatocytes of rats (Brown et al., 1979; Zammit and Easom, 1987). The negative effect of fasting on the concentration of HMG-CoA reductase in liver is believed to be mediated by the low concentration of insulin in serum (Zammit and Easom, 1987). The effect of insulin on activity of HMG-CoA reductase in bovine ovarian tissue has not been determined. However, exposure of luteinized porcine granulosa cells to insulin increased total HMG-CoA reductase activity by 70% (Veldhuis et al., 1986). Similarly, insulin increased

total HMG-CoA reductase activity in human fibroblasts (Brown et al., 1974). Therefore it is possible that the reduced concentration of insulin in serum noted during negative energy balance may result in reduced concentration of HMG-CoA reductase in bovine luteal tissue. If *de novo* synthesis of cholesterol is an important factor in luteal cell steroidogenesis, reduced concentration of HMG-CoA reductase could result in reduced luteal function.

Source of Acetyl-CoA for Cholesterol Synthesis

Substrate availability may affect the production of cholesterol for use in steroidogenesis. The production of acetoacetyl-CoA from two molecules of acetyl-CoA in the cytosol is an early step in the synthesis of cholesterol. There are many potential sources of acetyl-CoA in the cytosol of gonadal cells. Acetyl-CoA may be derived from acetate delivered by blood and activated by acetyl-CoA synthase in the cytosol. In addition, metabolism of glucose and various amino acids, oxidation of fatty acids, and conversion of 3-hydroxybutyrate and acetoacetate by the sequential action of 3-ketoacid CoA-transferase and acetoacetyl-CoA thiolase can yeild acetyl-CoA.

Acetate (Savard and Casey, 1964; Savard et al., 1965; Armstrong and Black, 1968; Armstrong et al., 1970; Pate and Condon, 1989) and glucose (Armstrong and Black, 1966) are incorporated into cholesterol and progesterone in ovarian tissue of rats and luteal tissue of cows (Flint and Denton, 1969; Anderson and Dietschy, 1979). In addition, the fatty

acid octonate can be incorporated into cholesterol in a variety of tissues of rats, including ovaries (Anderson and Dietschy, 1979). However, a role for free fatty acids in the production of progesterone in cattle has not been reported. A variety of tissues in ruminants including muscle, heart and kidney utilize ketones for production of acetyl-CoA (Bell, 1981; Bruss, 1989). Thus, there is a possibility that ketones may be used for precursors of ovarian steroids.

The relative importance of various precursors to *de novo* synthesis of cholesterol within bovine luteal tissue is not clear. However, there is evidence that some of these substrates may make a larger contribution to synthesis of cholesterol than do others. Acetate, a major product of carbohydrate fermentation in the rumen, may be an important source of acetyl-CoA in bovine CL. Approximately 70% of radioactively labeled acetate taken up by luteal tissue of rats is converted to fatty acids, steroids or sterols (Flint and Denton, 1969). The majority of radioactively labelled acetate metabolized by bovine luteal tissue is also converted to fatty acids, triglycerides, cholesterol or progesterone (Armstrong and Black, 1966; Armstrong et al., 1970). In fact, cholesterol and progesterone accounted for 90% of the total counts incorporated from labelled acetate (Armstrong et al., 1970). In contrast to acetate, production of fatty acids, sterols and steroids from glucose accounted for only 0.9% of the glucose taken up by the ovary (Flint and Denton, 1969). In cattle, 44% of the glucose that was incorporated by

tissue was converted to lipids (Armstrong and Black, 1966). However, similar to luteal tissue of rats, more glucose is metabolized to CO_2 than is converted to lipids in luteal tissue of cattle (Armstrong and Black, 1966). Bauman and Davis (1975) estimated the mammary gland of ruminants incorporates 125-fold more acetate than glucose into fatty acids. Therefore, If precursors for cholesterol synthesis in steroidogenic tissue are used similar to precursors for fatty acid synthesis in the mammary gland, acetate, not glucose, is the primary precursor for acetyl-CoA (Ingle et al., 1972).

Though acetate may be more important than glucose in supplying acetyl-CoA subunits for synthesis of cholesterol, glucose may play an important role in bovine luteal function. Gonadotropins affect the uptake and use of glucose by gonadal tissue. In luteal tissue of rats, LH stimulated the activity of enzymes involved in glycolysis including hexokinase and phosphofructokinase. In addition, LH increased the activity of enzymes in the pentose phosphate pathway, including glucose-6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Flint and Denton, 1969). Similarly, LH stimulated glucose uptake and glycolysis in luteal tissue of cows (Armstrong and Black, 1966; Armstrong et al., 1970). It is interesting to note that glucose uptake was higher in bovine CL actively synthesizing progesterone than in regressing CL (Armstrong and Black, 1966).

Obviously, the quantitative importance of glucose and acetate to ovarian steroidogenesis is not known. But both

compounds can be used by ovarian tissue to synthesize steroids and the concentration of both in serum are affected by energy balance. Following a one day fast, concentration of acetate in blood of cows declined from 1.55 mM to .38 mM (Lomax and Baird, 1983). Though less dramatic, concentration of glucose in blood is reduced in animals which are underfed, falling from 4.2 to 3.6 mM in plasma of starved heifers (McCann and Hansel, 1986) and from 3.0 to 2.7 mM in plasma of underfed lactating cows (de Boer et al., 1985). Reduced concentration of substrates used for *de novo* cholesterol synthesis may influence the production of progesterone. Therefore, during negative energy balance, reduced concentration of glucose or acetate may reduce luteal function.

Concentration of ketones in blood increase during negative energy balance. Specifically, concentration of acetoacetate increased from .03 to .15 mM in blood of non-lactating cows during a 6 day fast (Lomax and Baird, 1983) and from .28 mM to .49 mM in plasma of underfed lactating cattle (de Boer et al., 1985). In addition, 3-hydroxybutyrate which ranged between .27 mM (Bruss, 1989) to .99 mM (Hart et al., 1978) in plasma of dry cows increased to .88 mM in blood (Lomax and Baird, 1983) to 1.52 mM in plasma (de Boer et al., 1985) of underfed non-lactating or lactating cows, respectively. If ketones are used for synthesis of cholesterol by ruminant luteal tissue, increased concentration of ketones in blood may in part compensate for decreased concentration of acetate and glucose during negative energy

balance.

Metabolism of glucose, fatty acids and ketones produce acetyl-CoA in mitochondria. Acetyl-CoA must be in the cytosol to be used in the synthesis of cholesterol. Thus, acetyl-CoA from metabolism of glucose, fatty acids or ketones must be transferred out of the mitochondrion for use in synthesis of cholesterol. Acetyl-CoA is transferred across mitochondrial membranes as citrate. Citrate must then be cleaved by ATP-citrate lyase in the cytosol to yeild oxalacetate and acetyl-CoA (Bartley, 1989; Bruss, 1989). Energy balance may affect the activity of citrate lyase. The concentration of citrate increased in a variety of tissues of rats either when concentration of insulin was low, or following a 48 h fast (Parmeggiani and Bowman, 1963). A high concentration of citrate in tissue is consistent with reduced ability of tissue to metabolize citrate. Conversely, following a 4 h infusion of glucose, citrate lyase activity in liver of dry cows increased 1.5 fold (Bartley, 1989). Thus, it is possible that energy balance may affect precursor availability and may also affect the ability of tissue to utilize acetyl-CoA generated in the mitochondria for synthesis of cholesterol. Regardless of energy balance, the activity of citrate lyase in ovarian tissue has not been determined.

Cholesterol Side Chain Cleavage (C_{scc}) and Steroidogenesis

The cholesterol side chain cleavage enzyme system (C_{scc}) catalyzes the rate limiting step of steroid biosynthesis, the conversion of cholesterol to pregnenolone in the mitochondrion

(Strauss et al., 1981 for review). The three components of the C_{scc} enzyme system which participate in the oxidative cleavage of cholesterol include; $P\text{-}450_{scc}$, present on the inner mitochondrial membrane (Farkash et al., 1986), adrenodoxin, a non-heme iron containing protein, and adrenodoxin reductase. In addition to a source of cholesterol, molecular oxygen and NADPH are required for the reaction (McIntosh et al., 1971; Uzgiris et al., 1971; Caron et al., 1975; Funkenstein et al., 1983; Hall, 1984). Activity or amount of any of these components may affect activity of C_{scc} and thus synthesis of progesterone. There are many possible ways energy balance may affect the activity of the C_{scc} enzyme system in bovine luteal tissue. For example, energy balance may affect the concentration of reducing equivalents in luteal tissue. In addition, energy balance may affect the type of luteal cells present in the CL, and thus alter the concentration of C_{scc} in luteal tissue. Energy balance may also affect the intracellular and intramitochondrial transport of cholesterol. The effect of energy balance on the C_{scc} enzyme system and the conversion of cholesterol to pregnenolone, has not been investigated.

Source of Reducing Equivalents for C_{scc}

There are many possible sources of NADPH reducing equivalents in mitochondria of luteal cells. For example, production of NADPH from a variety of citric acid intermediates including succinate, malate and isocitrate can support C_{scc} activity in mitochondria from bovine CL (Uzgiris

et al., 1971). In addition, glucose metabolized in the pentose phosphate pathway may serve as a source of NADPH.

Glucose may supply reducing equivalents for cholesterol biosynthesis much in the same way glucose supplies NADPH for fatty acid biosynthesis in the mammary gland of ruminants (Bauman and Davis, 1975; Chaiyabutr et al., 1980). Addition of glucose to media increased incorporation of acetate into fatty acids 3 fold in adipose tissue of ruminants (Ingle et al., 1972). Similarly, addition of glucose increased incorporation of acetate into steroid in rat luteal tissue (Flint and Denton, 1969). Energy balance may affect the availability of NADPH from metabolism of glucose in the pentose pathway. Approximately 17% of glucose metabolized by the mammary gland of lactating goats is via the pentose phosphate pathway, and accounts for as much as 34% of the NADPH used in fatty acid synthesis (Chaiyabutr et al., 1980). However, following a 48 h fast there was an 8 fold increase in the percent of glucose metabolized to CO_2 and only 1.2% of the glucose used was metabolized by the pentose phosphate pathway (Chaiyabutr et al., 1980).

As mentioned earlier, restricted feeding heifers (McCann and Hansel, 1986) and lactating cows (de Boer et al., 1985) reduced the concentration of glucose in plasma. In addition, negative energy balance reduced the concentration of insulin in serum of cattle. Insulin stimulated glucose uptake in a variety of tissues in ruminants including muscle (Hay et al., 1984), fat (Vernon et al., 1985), and bovine granulosa cells

(Allen et al., 1981). As a result of reduced concentration of glucose and insulin in blood, negative energy balance may reduce uptake of glucose by luteal cells. Since glucose may be an important source of NADPH to luteal cells, reduced uptake of glucose could limit cholesterol synthesis in luteal cells. The effect of energy balance on glucose uptake by bovine luteal cells is not known.

Content and Activity of P-450$_{scc}$ and Adrenodoxin

The concentrations of P-450$_{scc}$ and adrenodoxin are low in bovine granulosa cells, but luteinization increases the concentrations 12 to 15 fold, respectively (Funkenstein et al., 1983; Rodgers et al., 1986b). Maximal concentrations of proteins and mRNA for P-450$_{scc}$ and adrenodoxin are concomitant with peak luteal function and then concentrations decline dramatically during the late luteal phase of cows (Rodgers et al., 1986b; Rodgers et al., 1987b). Thus, concentration of the components of the C$_{scc}$ enzyme in luteal cells is associated positively with maximal production of progesterone.

Concentration of P-450$_{scc}$ in luteal cells is affected by the type of luteal cell. Contentration of P-450$_{scc}$ is higher in large than in small luteal cells of rats (Gibori et al., 1988). Similarly, concentration of P-450$_{scc}$ was higher in large luteal cells compared with small luteal cells in cattle (Rodgers et al., 1986a). Rodgers et al. (1986a) suggested that the increased concentration of P-450$_{scc}$ in large luteal cells is due to increased cytoplasmic volume occupied by mitochondria in large luteal cells (Priedkalns and Weber,

1968a). Increased concentration of P-450$_{scc}$ may in part account for the higher production of progesterone per cell in large luteal cells compared with small luteal cells. Negative energy balance reduced the ratio of large luteal cells to small luteal cells in heifers (Villa-Godoy et al., 1990). Thus, negative energy balance may reduce the concentration of P-450$_{scc}$ in CL, and thereby reduce the production of progesterone by luteal cells.

Gonadotropins affect activity of the C_{scc} enzyme system. Addition of hCG to rat luteal cells and luteinized porcine granulosa cells, and addition of LH, cAMP or dbcAMP to bovine luteal tissue increased incorporation of cholesterol into progesterone in these tissues (Mason and Savard, 1964; Hall and Koritz, 1965; Savard et al., 1965; Armstrong and Black, 1968; Tan and Robinson, 1977; Azhar and Menon, 1981; Lino et al., 1985; O'Shaughnessy et al., 1990). Gonadotropins increase C_{scc} activity in part by increasing the concentration of the the various components of the C_{scc} system in steroidogenic tissue. Gonadotropins and analogs of cAMP increased the synthesis and activity of P-450$_{scc}$ concomitant with increased production of progesterone by luteinized bovine granulosa cells (Funkenstein et al., 1983, 1984). Similarly, hCG, FSH and cAMP increased the concentration of P-450$_{scc}$, P-450$_{scc}$ mRNA, adrenodoxin and adrenodoxin reductase in rat luteal tissue (Trzeciak et al., 1986; Goldring et al., 1987). Luteal cells from animals in negative energy balance were less responsive to stimulation by LH (Villa-Godoy et al., 1990).

Though the mechanism by which negative energy balance reduced LH-stimulated luteal function is not known, it is possible that negative energy balance may reduce the concentration of receptors for LH on luteal cells. As a result of reduced ability to respond to LH, the concentration of $P\text{-}450_{scc}$ in luteal tissue may be reduced. The effect of energy balance on the concentration of receptor for LH and the activity of the C_{scc} enzyme system has not been reported.

Transport of Cholesterol to C_{scc}

Biosynthesis of steroid requires the transport of cholesterol to the mitochondrion and transport across the outer and inner mitochondrial membranes to reach the C_{scc} enzyme system. The rate of steroid synthesis may be limited by the rate of transport of cholesterol to mitochondria and across mitochondrial membranes.

The majority of free cholesterol within cells is found in the plasma membrane (Lange and Ramos, 1983; Lange et al., 1988). The concentration of free cholesterol in the plasma membrane can affect steroidogenesis. For example, increasing the concentration of cholesterol in the plasma membrane increased basal and ACTH stimulated steroid production in adrenal cells (Iida et al., 1987). Cholesterol from intracellular cholesterol esters can also be used in steroidogenesis. However, the majority of cholesterol mobilized from cholesterol esters is first transported to the plasma membrane before it is used to produce progesterone (Nagy and Freeman, 1990). Cholesterol in the plasma membrane

does not appear to be the preferred substrate for steroidogenesis, however. Lange and coworkers (1988) differentially labelled cholesterol in luteinized rat granulosa cells to distinguish cholesterol associated with the plasma membrane and newly synthesized cholesterol in the cytosol. Following stimulation by gonadotropins, newly synthesized cholesterol was used preferentially over plasma membrane cholesterol in the production of progesterone (Lange et al., 1988).

Newly synthesized cholesterol may be used preferentially for steroid synthesis because cholesterol in the plasma membrane must first be transferred to the cytosol for uptake by mitochondria. The rate of transfer of cholesterol from the plasma membrane to mitochondria in steroidogenic tissue is controlled by hormones. Addition of ACTH or dbcAMP stimulated production of pregnenolone in adrenal cells and stimulated cholesterol transfer from the plasma membrane to mitochondria (Hall, 1985; Privalle et al., 1987; Iida et al., 1989). Similarly, dbcAMP reduced the concentration of cholesterol in the plasma membrane in MA-10 Leydig cells (a progesterone secreting tumor cell line) and increased production of progesterone reciprocally (Freeman, 1987, 1989). Hormonally controlled transfer of cholesterol to the mitochondria appears to require the cytoskeletal network. Compounds which interfere with the cytoskeleton decreased ACTH induced transport of cholesterol to mitochondria and decreased production of pregnenolone in adrenal cells (Hall, 1984,

1985). In addition, compounds which disrupt the network of microfilaments or microtubules of the cytoplasmic skeleton decreased the ability of hCG and dbcAMP to: stimulate incorporation of cholesterol esters into progesterone; decrease concentration of cholesterol in the plasma membrane; increase production of progesterone in MA-10 cells (Nagy and Freeman, 1990). Cytochalasin B, an inhibitor of microfilament function, decreased the ability of LH or dbcAMP to stimulate progestin synthesis in bovine luteal cells and slices of ovine luteal tissue (Williams and Marsh, 1979; Silavin et al., 1980). Gonadotropins seem to promote intracellular transport of cholesterol by altering the conformation of the cytoskeleton. Addition of ACTH or cAMP decreased the phosphorylation of myosin, increased polymerization of actin, and increased phosphorylation of proteins associated with the cytoskeleton of adrenal cells (Hall, 1985). These effects are associated with the contraction of the microfilament network and increased transport of compounds in cells (Means and Chafouleas, 1982; Goshima et al., 1984).

To determine if transport of cholesterol across mitochondrial membrane limits steroidogenesis, researchers have used 25-OH cholesterol, a compound which freely diffuses into the mitochondrion. Addition of 25-OH cholesterol increased production of steroids in adrenal tissue of rats, granulosa and luteal cells of hamsters, luteinized granulosa cells of pigs, and luteal cells of cows (Silavan and Strauss, 1983; Lino et al., 1985; Rodgers et al., 1987a; Iida et

al., 1989). Thus, it appears that transport of cholesterol across mitochondrial membranes limits the rate of steroidogenesis in bovine luteal tissue.

Transport of cholesterol from the outer to the inner mitochondrial membrane is sensitive to hormones. Addition of ACTH to adrenal cells and addition of hCG to luteinized rat ovaries increased the transport of cholesterol from outer to inner mitochondrial membranes (Hall, 1985; Ghosh et al., 1987; Privalle et al., 1987). Hormonally stimulated increased intramitochondrial transport of cholesterol is mediated by newly synthesized proteins. Exposure of animals to cycloheximide prior to ACTH, hCG, or LH blocked protein synthesis, increased the accumulation of cholesterol in the outer mitochondrial membrane, decreased C_{scc} activity and reduced production of progestins (Pederson and Brownie, 1983; Silavan and Strauss, 1983; Ghosh et al.,1987; Privalle et al., 1987).

Treatment with ACTH, hCG or LH induced synthesis of a variety of proteins in adrenal cortical cells and ovarian cells (Landefeld et al., 1979; DuBois et al., 1981a, 1981b; Trzeciak et al., 1986; Gibori et al., 1988; Yanagibashi et al., 1988; Mittre et al., 1990). Some of these proteins demonstrate the ability to facilitate transport of cholesterol from outer to inner mitochondrial membranes. For example, sterol carrier protein 2 (SCP2) participates in the microsomal conversion of lanosterol to cholesterol and intracellular transfer of cholesterol from outer to inner mitochondrial

membranes in hepatocytes (Scallen and Vahouny, 1985). Sterol carrier protein 2 has been isolated from adrenocortical and luteal cells of rats (Trzeciak et al., 1987; Gibori et al., 1988). In steroidogenic cells, the majority of SCP2 is located in mitochondria (Gibori et al., 1988). Thus, the major role of SCP2 in steroidogenic tissue may be in intramitochondrial transport of cholesterol. Treatment with ACTH or dbcAMP increased synthesis of SCP2 in adrenal cells 3-4 fold (Trzeciak et al., 1987). Addition of SCP2 to isolated adrenal mitochondria increased incorporation of cholesterol into pregnenolone in a dose dependent fashion (Scallen and Vahouny, 1985). Similarly, estradiol increased mitochondrial SCP2 in rat luteal cells 3 fold and stimulated the production of progestins (Gibori et al., 1988). Thus, cholesterol transport is another factor of steroidogenic function which is under hormonal control and may be modulated to control luteal function.

Summary of the Relationship between Energy Balance and Bovine Luteal Cell Function

Negative energy balance is associated with reduced concentration of progesterone in serum of cows and heifers. The majority of dairy cattle experience negative energy balance early in lactation. This period of negative energy balance coincides with the period in which animals must conceive in order to achieve a 12 to 13 month calving interval. Reduced concentration of progesterone in serum may

reduce ability to achieve and maintain pregnancy. Therefore, it is important to understand the mechanisms by which negative energy balance reduces the concentration of progesterone in serum of cattle in order to alleviate this limitation and to achieve reproductive goals in dairy herds.

Change in development of CL is one mechanism by which negative energy balance may reduce concentration of progesterone in serum. For example, negative energy balance can reduce the weight of CL in cattle. The cause for reduced weight of CL in animals in negative energy balance has not been determined. However, it is possible that reduced weight of CL is caused by decreased concentration of LH in serum during the periovulatory period in heifers in negative energy balance. LH is associated positively with hypertrophy and hyperplasia of luteal cells and vascular development of luteal tissue. As a result of reduced concentration of LH in serumduring the periovulatory period, growth and development of the CL may then be inhibited during negative energy balance.

Energy balance of cattle may also affect luteal function by changing the proportion of large luteal cells in CL. Negative energy balance appears to reduce the ratio of large to small luteal cells. Compared with luteal cells from heifers in positive energy balance, negative energy balance reduced basal and LH-stimulated production of progesterone *in vitro*. Reduced basal production of progesterone is consistent with the reduced ratio of large to small luteal cells from

animals in negative energy balance. However, reduced ability of LH to stimulate production of progesterone is inconsistent with a reduced ratio of large to small luteal cells. Compared with large luteal cells, small luteal cells have a higher concentration of receptors of LH and are normally more responsive to stimulation by LH. It is possible that the concentration of receptors for LH on small luteal cells is reduced during negative energy balance. However, the effect of energy balance on concentration of receptors for LH has not been determined.

Metabolism of cholesterol is central to production of progesterone by luteal tissue. Cholesterol in bovine luteal tissue is derived from lipoproteins in blood and *de novo* synthesis in luteal tissue. Negative energy balance may reduce concentration of cholesterol in luteal tissue from either source. Low density lipoprotein may be the preferred carrier for delivery of cholesterol to bovine steroidogenic tissue. There is some evidence that restricted energy intake decreases the concentration of LDL in blood of ruminants. In addition, decreased concentration of insulin and insulin like growth factor I in blood of cattle during negative energy balance may reduce the concentration of receptors for LDL on luteal tissue. As a result of reduced concentration of LDL in blood and reduced concentration of receptors for LDL on luteal cells during negative energy balance, the concentration of cholesterol in luteal tissue may be reduced. Negative energy balance may also reduce *de novo* synthesis of cholesterol in

luteal cells. There are several ways negative energy balance may affect activity of HMG-CoA reductase in luteal tissue. These possibilities include decreased precursor availability in blood, decreased uptake of precursors from blood by luteal cells, and decreased activity of HMG-CoA reductase in luteal cells.

Negative energy balance may decrease convertion of cholesterol into progesterone by the C_{scc} enzyme system in luteal cells. Conversion of cholesterol to progesterone is affected by transport of cholesterol to the inner mitochondrial membrane, concentration of reducing equivalents and O_2, and activity of the components of the C_{scc} enzyme complex. Negative energy balance may reduce intracellular transport of cholesterol, reduce concentration of reducing equivalents and reduce activity of the C_{scc} enzyme system in luteal cells. It is, therefore, of interest to evaluate the effect of energy balance of the ability of luteal cells to convert cellular cholesterol to progesterone.

Clearly, energy balance may affect luteal function by a variety of mechanisms. For this thesis I focused primarily on the effect of energy balance on; 1) concentration of receptors for LH on luteal cells, 2) concentration of free and esterified cholesterol in luteal cells, 3) total activity of HMG-CoA reductase in CL, 4) and ability of luteal cells to convert cholesterol into progesterone.

EXPERIMENT I: INFLUENCE OF RESTRICTED ENERGY INTAKE ON CONCENTRATION OF RECEPTORS FOR LH AND METABOLISM OF CHOLESTEROL IN BOVINE LUTEAL CELLS

Rationale and Objectives for Experiment I

Compared with luteal cells from animals in positive energy balance, negative energy balance reduced basal and LH-stimulated production of progesterone by bovine luteal cells (Villa-Godoy et al., 1990). Negative energy balance also reduced weight of CL and reduced the proportion of luteal cells that are large in bovine CL (Villa-Godoy et al., 1990). Luteinizing hormone is the primary luteotropin in cattle and affects both development and function of CL. In the simplest scenario, energy balance may affect luteal production of progesterone by altering the number of receptors for LH on luteal tissue. My first objective was to determine the effect of energy balance on the number of receptors for LH on bovine luteal cells.

Cholesterol is required for production of progesterone. Increased availability of cholesterol enhances bovine luteal production of progesterone *in vivo* and *in vitro*. Therefore, factors which influence the concentration of cholesterol in luteal tissue could also affect the production of progesterone. Energy balance may alter both luteal uptake of lipoprotein derived cholesterol and *de novo* synthesis of

cholesterol by luteal tissue. Thus, my second objective was to determine if energy balance affects the concentration of cholesterol in luteal cells.

Energy balance may affect luteal function by altering several steps in the conversion of cholesterol to progesterone. My third objective was to determine the acute effect of energy balance on metabolism of cholesterol in bovine luteal cells *in vitro*.

Materials and Methods

Materials

The following reagents were used; cholesterol esterase (from Candida cylindracea), cholesterol oxidase (from Nocardia erthropolis), horseradish peroxidase (grade 1), Triton X-100, Boehringer Mannheim Biochemicals (Indianapolis, IN); penicillin-G, streptomycin sulfate, cholesterol (Sigma grade), cholesteryl oleate (Sigma grade), p-hydroxyphenylacetic acid, taurocholic acid (sodium salt), deoxyribonuclease 1 type II (DNase 1), minimum essential media with non-essential amino acids and Hank's salts (MEM), N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), trypan blue, bovine serum albumin fraction V (BSA), Sigma (St. Louis, MO); isopropyl alcohol (high purity solvent), American Burdick and Jackson (Muskegon, MI); methanol (HPLC grade) Fisher Scientific (Fairlawn, NJ); chloroform, J.T. Baker Inc. (Phillipsburg, NJ); collagenase type IV, Worthington Biochemical Corporation (Freehold, NJ); phenol reagent, Harleco (Gibbstown, NJ);

prostaglandin $F_{2\alpha}$ (PGF; Lutylase®), The Upjohn Company, (Kalamazoo, MI); xylazine hydrochloride (Rompun®) Mobay Corporation (Shawnee, KS); 25 cm^2 tissue culture flasks, Corning Glass Works (Corning, NY); 12 x 75 mm borosilicate tubes, VWR Scientific Inc. (San Francisco, CA); 12 x 75 mm sterile polypropylene tubes, Falcon (Oxnard, CA). Others are as cited in text.

Animals

Sixteen postpubertal Holstein heifers were assigned randomly to one of two diets (Table 3). A three week period in which all heifers were fed the Control diet was used to acclimate heifers to group housing (4 pens with 4 heifers/pen). During this 3-week period, heifers received 2 injections of PGF spaced eleven days apart to synchronize estrous cycles. Feed was offered to pens of heifers once daily at 1100 h. The Control diet was designed to supply enough energy for maintenance and growth of heifers in a pen, while the Restricted diet was designed to restrict energy intake below requirements for maintenance of heifers in a pen. Otherwise, the diets met National Research Council (NRC, 1978) requirements for protein, vitamins and minerals for maintenance (Restricted) or maintenance plus growth (Control). Amount of feed offered each pen was based on the body weight of heifers in the pen and desired energy intake for those heifers.

One to three days after observation of the first synchronized estrus (estrus=day 0), the Restricted diet was

Table 3. Feed Offered Per Heifer[a]

Diet	Week fed[b]	Dietary components: Corn silage (kg)	Soy bean meal (kg)	Wheat straw (kg)	Vitamin mineral mix[c] (kg)	NEM[d] (Mcal)	NEG[e] (Mcal)
Control	1 to 6	5.67	.70	-	.09	6.24	2.42
Restricted	1 to 3	.95	1.40	1.00	.11	5.15	-
	3 to 6	.64	1.41	-	.11	3.65	-

[a]Data represent amount of feed offered on a dry matter basis to an average heifer weighing 350 kg. Heifers were feed in groups of four, and pens were bedded with straw. Thus, actual dry matter intake of a heifer could not be determined.

[b]Week relative to first synchronized estrus. Week 1 = Week following synchronized estrus. In addition, all heifers received the Control diet for three weeks before first synchronized estrus.

[c]Vitamin mineral mix composition per kg: limestone, .375 kg; trace mineral salt, .281 kg; selenium 200 (200 ppm), .143 kg; calcium sulfate, .009 kg; 177.4 KIU vitamin A; 35.71 KIU vitamin D; 3.99 KIU vitamin E.

[d]Net energy for maintenance

[e]Net energy for gain

offered heifers in two of the four pens. All heifers were weighed on two consecutive days each week and amount of feed offered was adjusted according to changes in body weight.

Estrous behavior and concentration of progesterone in serum were used to monitor luteal function and length of estrous cycles. Heifers were observed three times daily for estrous behavior. Jugular blood was sampled daily starting on the day after the first synchronized estrus to determine the concentration of progesterone in serum. Estrous behavior and concentration of progesterone in serum were also used to determine length of estrous cycles following estrous synchronization. A normal estrous cycle was defined as one with an inter-estrous interval of 17 to 25 days, progesterone in serum ≥1 ng/ml for ≥13 days, and ended with detection of standing estrus (King, 1984).

Tissue

Heifers were tranquilized with xylazine hydrochloride (1 mg/100 lb body weight iv) on day 11, 12, or 13 postestrus (12.1 ± .1 day) of the second or third estrous cycle following estrous synchronization. Upon collection per vaginum, CL were rinsed with and then stored in ice cold Hank's balanced salt solution (HBSS with 20 mM HEPES, .1% BSA, 100 U penicillin G/ml, 100 ug/ml streptomycin sulfate, pH 7.25) and transported immediately to the laboratory. After adherent connective tissue was removed, CL were weighed and then dissociated as described previously (Villa-Godoy et al., 1990). The final cell preparation (cell yield = 63 ± 4 x 10^6 cells/gm tissue)

was diluted with MEM to 1 x 10^6 cells/ml as determined via hemacytometer. Cell viability was estimated by exclusion of trypan blue and ranged from 90 to 95% among CL. Aliquots of cells were collected to determine initial concentration of progesterone, protein (Lowry et al., 1951), or cholesterol and for radioreceptor assays for LH. Cells saved for receptor analysis were suspended in 20% glycerol-MEM (vol:vol) frozen in a dry ice and methanol bath and stored at -70°C until assayed.

To examine luteal function and cholesterol metabolism, cells (1 x 10^6 per polypropylene tube) were incubated for 2 h at 37°C in a shaking water bath with 0 or 100 ng NIH-LH-B8 in 20 µl phosphate buffered saline (PBS). Following incubation, cells and media were separated by centrifugation at 800 x g. Media was saved for determination of progesterone, and cells were saved for determination of progesterone or cholesterol. Cells for determination of progesterone were suspended in 2 ml PBS. Cells and media were then stored at -20°C until analyzed.

Validation of LH Binding Assay

In our laboratory, assays to determine number and equilibrium association constants (K_a) of binding sites for hCG have been validated for homogenized luteal tissue (Spicer et al., 1981) but not for dispersed luteal cells. To validate radioreceptor assays for dispersed luteal cells, a pool of dissociated cells was prepared from CL of nonpregnant heifers. In addition, a pool of homogenized luteal tissue was prepared

from CL collected from nonpregnant cattle at slaughter as described previously (Spicer et al., 1981). All tissue for radioreceptor assay validation was stored frozen in 20% glycerol-PBS at -70°C until day of use. To test for specificity of binding sites in luteal cells to ^{125}I-hCG, luteal cells were incubated with ^{125}I-hCG in the presence of unlabeled hCG. To determine if the number of cells which were incubated affected binding of ^{125}I-hCG, .2 x 10^6 to 6 x 10^6 cells were incubated with ^{125}I-hCG (20,000 cpm). To estimate effects of luteal tissue preparation and number of luteal cells on K_a and numbers of unoccupied binding sites, Scatchard analyses were performed with 5 mg luteal homogenates, .25 x 10^6 luteal cells and 1 x 10^6 luteal cells (Smith and Sestili, 1980). Specific data are reported in Appendix 1. But, salient results were that assays for LH receptors on luteal cells demonstrated saturable binding of high specificity and affinity for hCG. In addition, type of tissue preparation and number of luteal cells did not affect estimates of K_a or number of receptors for LH.

Procedure for LH Binding Assay

Human chorionic gonadotropin (hCG, CR-121; 13450 IU/mg) was radioiodinated as described previously (Spicer et al., 1981). Specific activity of ^{125}I-hCG was estimated by incorporation of iodine into the mass of hormone (Ireland and Roche, 1982) and with autocompetition curves as by Spicer et al. (1981). Autocompetition curves were conducted with increasing amounts of unlabelled hCG (.1 to 100 ng/tube) or

^{125}I-hCG (.02 x 10^6 to 1.5 x 10^6 cpm) with 1 x 10^6 cells/tube. Results from the two types of analyses of specific activity were similar and averaged 92.2 cpm/µg hCG. Maximal specific binding (Spicer et al., 1981) to luteal tissue was 16% in frozen, pooled luteal homogenates. Calculations of specific activity and total radioactivity added in saturation analysis were corrected for maximal specific binding (counts per minute corrected, cpm_{crt}).

Binding assays were conducted in polypropylene tubes coated with PBS-5% BSA to reduce non-specific adsorption of ^{125}I-hCG. Frozen luteal tissue homogenates or dispersed luteal cells were thawed rapidly and rinsed three times with PBS. After each rinse, tubes were centrifuged at 2200 x g for 5 min at 4°C. Except for some procedures noted in the assay validation, luteal homogenates (5mg) or luteal cells (5.0 ± .3 x 10^5 cells) were suspended in 100 µl PBS. To distinguish specific versus non-specific binding, luteal homogenates and cells were incubated with 100µl ^{125}I-hCG at 25°C for 24 h in a shaking water bath in the presence or absence of excess LH (NIAMMD-LH-B4; 40 µg/20 µl PBS). To construct saturation curves, dispersed luteal cells from animals on experiment were incubated with 4 different concentrations of ^{125}I-hCG (3 to 13 x 10^5 cpm_{crt}) in quadruplicate either with or without excess LH. Final incubation volume was 220 µl. After incubation, 1 ml PBS at 4°C was added and tubes were centrifuged at 2200 x g for 15 min at 4°C. Supernatants were discarded and pellets were suspended in 1 ml PBS and the process repeated. The

final precipitate was counted in a gamma counter (counting efficiency = 83.2%). Scatchard plots constructed from saturation curves were used to estimate number and affinity of binding sites for LH in cells.

Quantification of Cholesterol

Luteal cells (1 x 10^6) were transferred to 12 x 75 mm borosilicate tubes and suspended in 2 ml chloroform:methanol (1:2, vol:vol). Tissue was sonicated for 10 x 1 sec bursts with a Branson® Sonifer Cell Disrupter (Ultrasonic Inc., Plainview, NY). Samples were centrifuged for 15 min at 800 x g and the supernatants were removed. Pellets were retained and analyzed for protein content. Solvents in supernatants were evaporated under nitrogen and the residue was resuspended in 500 µl isopropanol. Samples were then assayed for total cholesterol (unesterified and esterified cholesterol) or unesterified (free) cholesterol (Heider and Boyett, 1978). Concentration of esterified cholesterol was calculated as the difference between total and free cholesterol. The intraassay coefficients of variation were 6.4% and 6.7% and interassay coefficients of variation were 8.9% and 12.0% for total and free cholesterol, respectively.

Some changes in published methodology of Heider and Boyett (1978) were necessary. Amount of cholesterol ester hydrolase was increased to .16 U/ml and Triton X-100 (.016%) substituted for Carbowax-600 (personal recommendation, John Heider). Concentration of sodium taurocholate was increased to 16 mM to achieve parallel regression lines between

cholesterol and cholesterol oleate versus relative fluorescence. With these changes, the results from this assay were consistent. In addition, I determined that the number of luteal cells extracted (.25 x 10^6 to 1.0 x 10^6) did not affect estimates of concentration of cholesterol in luteal cells. Concentration of cholesterol in a pool of dispersed bovine luteal cells, averaged 6.0 µg/10^6 cells or 3 mg/gm dissociated cells. In the standard pool, 10^6 luteal cells weighed approximately 2 mg and contained 200 µg protein. Unesterified cholesterol accounted for 80% of total cholesterol present. These data agree closely with data from thin layer chromatography of extracts of bovine CL (Hafs and Armstrong, 1968).

Radioimmunoassay of Progesterone

Serum, collected after centrifugation of blood samples, was stored at -20°C until assayed for concentration of progesterone. Luteal cells, suspended in 2 ml PBS, were frozen and thawed 6 times to rupture plasma membranes. Concentration of progesterone in serum, media or cells was determined as described previously (Spicer et al., 1981), except cellular preparations were extracted twice. Coefficients of variation for assays of serum, media or cells were determined from pools of serum, media or cells. Coefficients of variation for these pools averaged 9.8% within and 12.1% between assays.

Statistical Analyses

Body weights at the beginning of the experiment, on the day of estrus at the beginning of cycle 2 and on the day of lutectomy were determined from weekly body weights. Daily change in body weight was determined for the first and second estrous cycle. To examine changes in luteal function *in vivo* during the first estrous cycle following estrus synchronization, area under the profile of progesterone in serum was calculated during the entire estrous cycle. To determine the effect of diet on luteal function during estrous cycle 1 and estrous cycle 2, area under the profile of of progesterone in serum was calculated for the first eleven days of cycle 1 and cycle 2.

One way analysis of variance was used to test for effect of diet on number and affinity of receptors for LH, weight of CL, length of estrous cycles and luteal function during the first estrous cycle. Differences between dietary groups were examined by Student's t tests. Split plot analysis of variance with time as the subplot was used to examine effect of diet and diet by time interactions on body weights. Similarly, split plot analysis of variance with estrous cycle as the subplot was used to examine the effect of diet and diet by cycle interactions on luteal function during estrous cycles 1 and 2. Split plot analyses of variance with incubation as the subplot was used to examine effect of diet and diet by incubation interaction on concentration of cholesterol in tissue and concentration of progesterone in tissue and media

(Gill, 1978b). For split plot analyses with significant interaction between diet and incubation, conditional comparisons of periods within treatments, treatments within periods and treatment trends between specified periods were examined with Bonferroni t tests (Gill, 1986). To determine if change in body weight during the entire experiment, or during cycle 1 or 2 affected variables of interest, change in body weight was used as a covariate in some analyses.

Degree of association among variables of interest within a dietary group was determined with Pearson correlation coefficients. Regression analysis was used to determine if the relationship between number of receptors for LH and weight of CL, or weight of CL and the concentration of progesterone in serum during estrous cycle 2 were affected by diet.

There was unequal variance between treatment means for length of estrous cycle 1 and between treatment means for concentration of free and total cholesterol. In these cases unequal variance could not be removed by conventional transformations of data (Hartley's Fmax test; Gill, 1978a). Contrasts of untransformed data regarding estrous cycle length were made using the modified f statistic of Brown and Forsythe (Gill, 1978a). Contrasts of untransformed data involving concentrations of free and total cholesterol were made following alterations in the test statistics as suggested by Gill (1986).

Data analyses were performed with mainframe SAS (1985a and b).

Results

Animals

One Control animal was deleted from the experiment after it contracted a respiratory disease. Immediately following estrous synchronization, four heifers had aberrant cycles. One heifer fed the Restricted diet had a cycle of 29 days. Concentrations of progesterone in serum from this heifer were <0.5 ng/ml for the first 15 days following synchronization. These 15 days were excluded from analyses of data. A normal luteal phase followed this 15 day period. This luteal phase was regarded as part of estrous cycle 1 for this animal. The rest of the data from this animal are included in analyses. Two Control and one Restricted animal had short estrous cycles of 10 to 12 days following estrous synchronization. In the two Control animals the subsequent estrous cycle was normal and was included in analyses as cycle 1 for these animals. Lutectomies of these two heifers were performed during the third estrous cycle after synchronization. The Restricted heifer had concentration of progesterone in serum indicative of normal luteal function during the experiment. However, this heifer was not observed in estrus. This animal was deleted from the experiment as day of the estrous cycle could not be determined accurately. As a result of deletions of animals, there were 7 heifers in each dietary group. Average length of the estrous cycle before lutectomy in Restricted heifers (22.4 ± 1.3 days) was more variable ($P \leq .01$) and tended ($P \leq .10$) to be longer than in Control heifers (20.1 ± .3 days).

Body Weight

Initial body weights did not differ between treatment groups (Figure 2). However, diet affected change in body weight during the experiment. Body weight of Control heifers increased ($P \leq .05$) during cycle 1 and cycle 2. In contrast, body weight of Restricted heifers did not change during the experiment. Weight change varied between estrous cycles and among animals in the Restricted group (Table 4), but heifers either lost weight (5/7 heifers), or gained less than .06 kg/day during the experiment. Change in body weight can be used as an approximation of energy balance. Using this approximation, Control heifers were in positive energy balance and Restricted heifers maintained energy balance.

Concentration of Progesterone in Serum

Area under the profile of progesterone in serum was numerically less in Restricted heifers than in Control heifers during estrous cycle 2 (Table 5). However, there was no significant effect of diet, cycle or diet by cycle interaction on the area under the profile of progesterone. As noted previously, change in body weight varied among animals in a treatment group and between estrous cycles. Change in body weight during the experiment was used as a covariate to account for this variation. During cycle 1, change in body weight accounted for a significant ($P \leq .03$) amount of the variation in the profile of progesterone. The correlation between change in body weight and the profile of progesterone during estrous cycle 1 tended ($P=.07$) to be positive ($r=.493$).

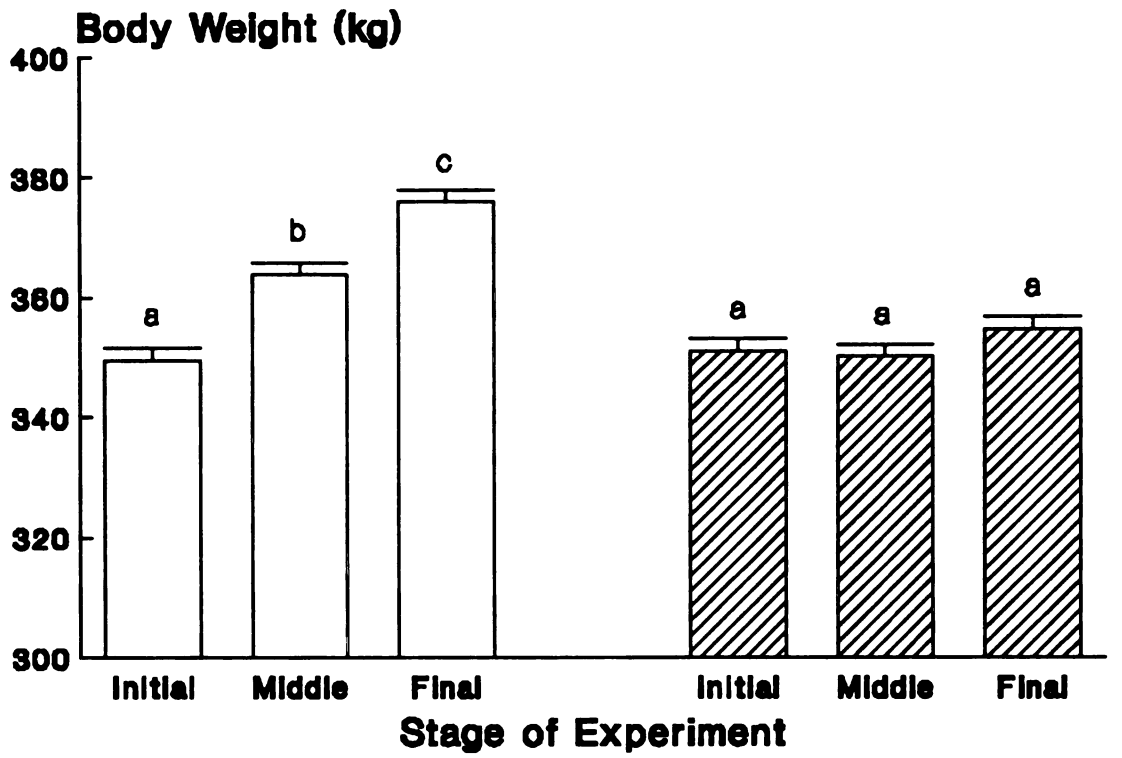


Figure 2. Effect of diet on body weights of heifers. Body weight at first synchronized estrus (Initial), at the beginning of cycle 2 (Middle), and at lutectomy (Final). Weights of Control heifers are represented by open bars. Weights of Restricted heifers are represented by hatched bars. Data are presented as means (+ pooled SEM). ^{abc}Bars with different superscripts differ ($P \leq .05$).

Table 4. Effect of Diet on Changes in Body Weight During Estrous Cycle 1 and 2.

Diet	Estrous Cycle	Change of Body Weight	
		Mean[a] (kg/day)	Range (kg/day)
Control	1	.61*	.23 to .98
	2	1.01*	.12 to 1.45
Restricted	1	-.06	-.65 to .46
	2	.38	-.17 to 1.04

[a]Pooled standard error of the mean = ± .19 kg.

*Change is different from zero (P≤.05).

In addition, change in body weight during estrous cycles 1 and 2 was correlated positively with the profile of progesterone during estrous cycle 2 in Restricted heifers ($r = .75$, $P \leq .05$), but not in Control heifers.

Weight of CL

Weight of CL in Restricted animals (4.96 gm) tended ($P \leq .08$) to be less than weight of CL in Control animals (6.16 gm; SEM = ± .44 gm). Change in body weight during estrous cycle 1 and 2 and change in body weight during cycle 2 did not affect weight of CL. Independent of dietary treatment, there was a significant positive linear relationship between weight of CL and the area under the profile of progesterone during estrous cycle 2 ($P \leq .04$). The correlation between weight of CL and the profile of progesterone in serum during estrous cycle 2 was $r=.677$ ($P \leq .008$).

Binding Sites for LH

Change in body weight did not affect the number or affinity of receptors for LH on bovine luteal cells (Table 6). However, diet tended to affect affinity and number of receptors for LH on luteal cells. Specifically, the Restricted diet tended ($P \leq .08$) to reduce the affinity and tended ($P \leq .10$) to increase the concentration of receptors for LH in luteal cells. Diet also affected the linear relationship between the concentration of receptors for LH and the weight of CL ($P \leq .001$). The concentration of receptors for LH on luteal cells was correlated positively ($r=.772$, $P \leq .04$) with weight of CL in Control animals, but not with weight of

Table 5. Effect of Diet on Concentration of Progesterone[a] in Serum of Heifers.

Diet	Estrous Cycle 1 (Entire Cycle) Progesterone[c] ($ng \cdot ml^{-1} \cdot day$)	CV[d] (%)	Estrous Cycle 1 (First 11 Days)[b] Progesterone[e] ($ng \cdot ml^{-1} \cdot day$)	CV (%)	Estrous Cycle 2 (First 11 Days) Progesterone ($ng \cdot ml^{-1} \cdot day$)	CV (%)
Control	25.6[c]	48.5	9.7[d]	42.6	10.5	42.9
Restricted	26.4	33.9	9.2	27.1	8.5	44.6

[a]Data are expressed as area under the profile of progesterone in serum.

[b]Day 1 to day 11 of the estrous cycle.

[c]Standard error of the mean for estrous cycle 1 = ± 4.1.

[d]Coefficient of variation within dietary group.

[e]Pooled standard error of the mean for day 1 to day 11 of estrous cycle 1 and 2 = ± 1.1.

Table 6. Effect of Diet on Affinity Constants (K_a) and Numbers of Unoccupied Binding Sites for ^{125}I-hCG on Dispersed Luteal Cells

Diet	Affinity Constant[a] ($x10^{10}M^{-1}$)	Number of Binding Sites[b] (fMol/mg protein)
Control	2.32	20.62
Restricted	1.51	31.76

[a]Means within the column differ ($P \leq .08$), SEM = ±.3.

[b]Means within the column differ ($P \leq .10$), SEM = ±4.39.

CL in Restricted animals (r=-.477, P≥.2).

Steroidogenic Function of Luteal Cells

Diet and change in body weight did not affect initial concentration of progesterone in tissue or concentration of progesterone in cells or media following incubation with 0 ng (Basal) or 100 ng LH (Stimulated; Figure 3). Progesterone increased (P≤.05) during the 2 h Basal incubation, but was higher (P≤.01) when LH was present in the media. Concentration of receptors for LH on luteal cells was correlated positively with LH-stimulated concentration of progesterone *in vitro* in Restricted (r=.942, P≤.002), and Control heifers (r=.883, P≤.009).

Luteal Cell Cholesterol Metabolism

Diet and incubation did not affect concentration of esterified cholesterol in luteal cells (Figure 4). However, change in body weight during cycle 2 affected concentration of esterified cholesterol in luteal cells (P≤.05). Change in body weight was correlated negatively with initial concentration of cholesterol esters (r=-.623, P≤.02). In addition, the correlation between initial concentration of cholesterol esters and the area under the profile of progesterone during estrous cycle 2 tended to be negative (r=-.482, P≤.08).

Diet did not affect the initial concentration of free or total cholesterol in luteal cells. However, there were significant interactions between diet and incubation on the concentration of free (P≤.04) and total (P≤.10) cholesterol in

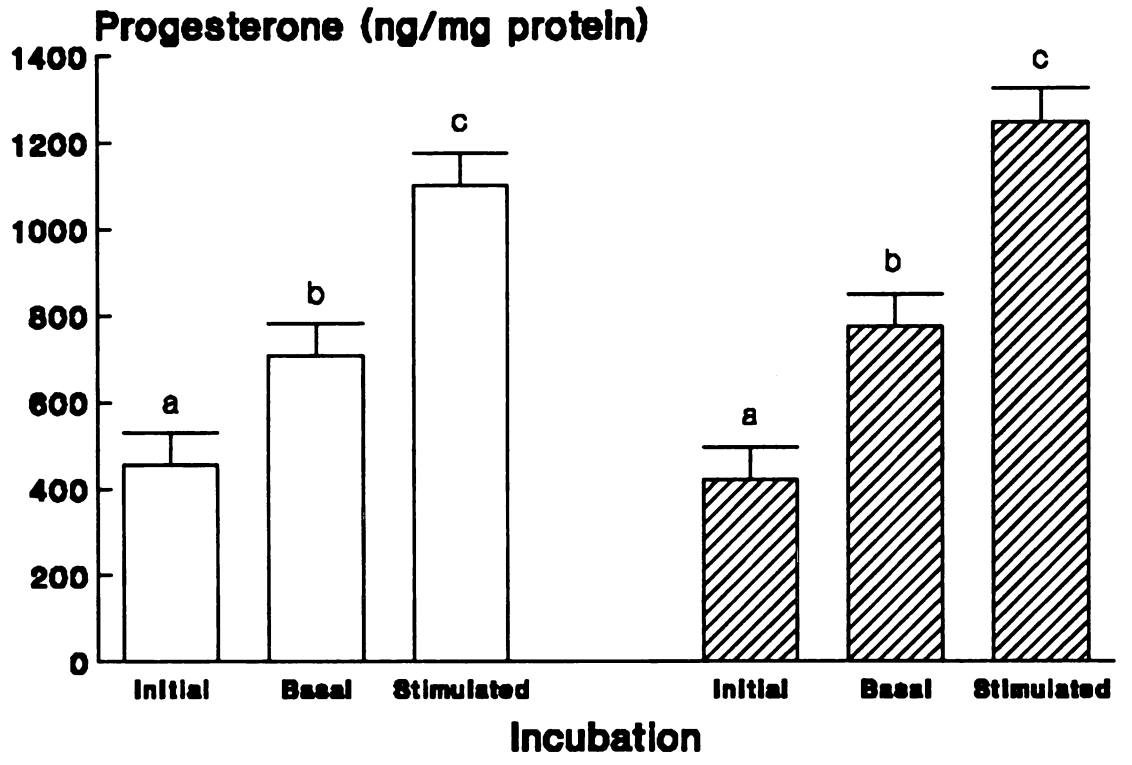


Figure 3. Effect of diet on concentration of progesterone in luteal cells and incubation media before incubation (Initial), and following a 2 h incubation with 0 ng LH (Basal), or with 100 ng LH (Stimulated). Data are presented as means (+ pooled SEM). Control animals are represented by open bars. Restricted animals are represented by hatched bars. ^{abc}Means within a diet with different superscripts differ ($P \leq .01$).

luteal cells. In luteal cells of Restricted heifers, there was no difference in the initial concentration of free or total cholesterol in luteal cells among Initial, Basal and LH-stimulated incubations. But, in luteal cells of Control heifers, the concentration of free ($P \leq .01$) and the concentration of total ($P \leq .02$) cholesterol increased between Initial and Basal incubations (Figure 5). In addition, luteal cells of Control heifers had lower concentrations of free and total cholesterol after incubation with 100 ng LH than in those incubated without LH ($P \leq .02$).

Interrelationships among concentration of free or esterified cholesterol, concentration of progesterone *in vitro*, and concentration of receptors for LH were examined by correlation (Table 7). In Restricted heifers, concentration of progesterone following incubation was correlated positively with Initial concentration of cholesterol esters and the loss of esterified cholesterol between Initial and Basal incubations. Similarly, LH-stimulated concentration of progesterone was correlated positively with loss of esterified cholesterol between Initial and LH-stimulated incubations. In luteal cells of Restricted heifers incubated with LH, the concentration of receptors for LH tended ($P \leq .10$) to be correlated positively with loss of esterified cholesterol.

In contrast to Restricted heifers in luteal cells of Control heifers, LH-stimulated concentration of progesterone was correlated positively ($P \leq .01$) with the difference in concentration of free cholesterol between Basal and

Figure 4. Effect of diet on concentration of free (unesterified), esterified and total (esterified and unesterified) cholesterol in luteal cells. Values represent means (+SE) of Initial concentration of cholesterol and concentration of cholesterol following a 2 h incubation with 0 ng LH (Basal), and with 100 ng LH (Stimulated). Control animals are represented by open bars, Restricted animals are represented by hatched bars. [ab]Within type of cholesterol and treatment group, bars with different superscripts differ ($P \leq .02$).

Figure 4.

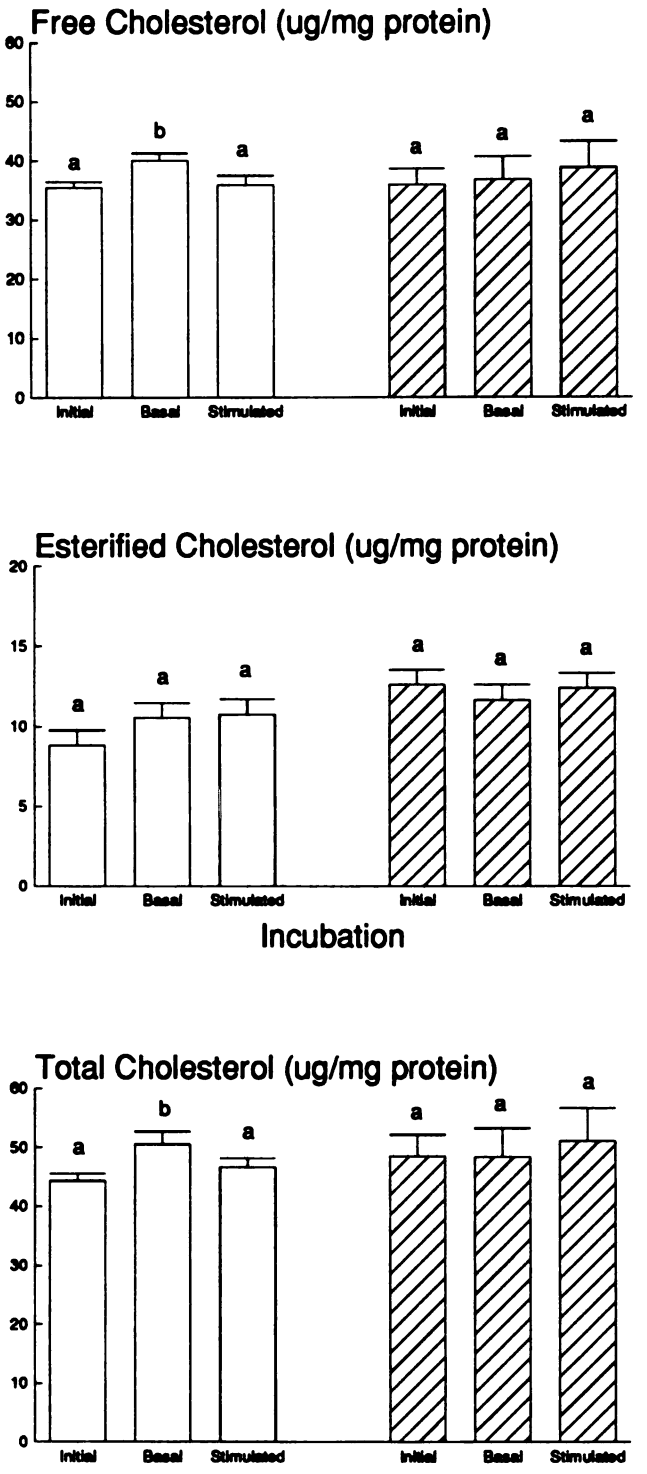
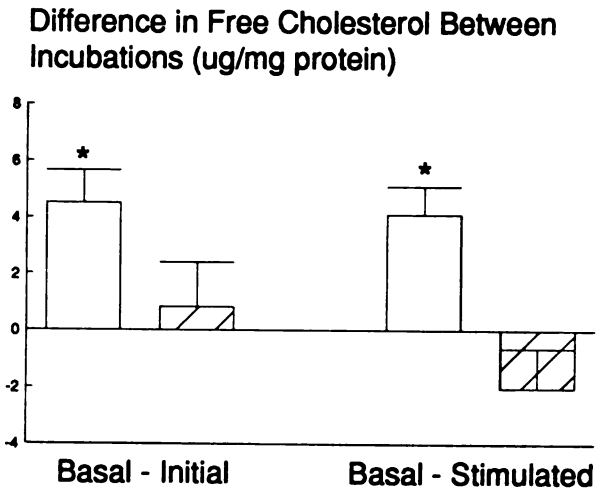
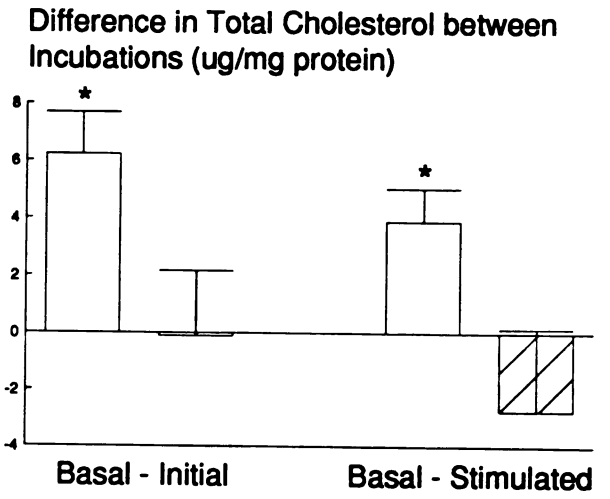

Free Cholesterol (ug/mg protein)
60
50
40
30
20
10
0
a
b
a
a
a
a
Initial
Basal
Stimulated
Initial
Basal
Stimulated
Esterified Cholesterol (ug/mg protein)
20
15
10
5
0
a
a
a
a
a
a
Initial
Basal
Stimulated
Initial
Basal
Stimulated
Incubation
Total Cholesterol (ug/mg protein)
60
50
40
30
20
10
0
a
b
a
a
a
a
Initial
Basal
Stimulated
Initial
Basal
Stimulated

Figure 5. Effect of diet on changes in concentration of free (unesterified) and total (esterified and unesterified) cholesterol in luteal cells. This figure is used to illustrate the effect of diet on the concentration of free and total cholesterol gained (+) or lost (−) between incubations. Incubations (Initial, Basal, Stimulated) are as described in Figure 4. Values represent the difference between incubations within treatments (+ standard error of the difference; SED). Control animals are represented by open bars. Restricted animals are represented by hatched bars. *Mean is different from 0 ($P \leq .02$).

Figure 5.


Difference in Free Cholesterol Between Incubations (ug/mg protein)
8
6
4
2
0
-2
-4
*
*
Basal - Initial
Basal - Stimulated


Difference in Total Cholesterol between Incubations (ug/mg protein)
8
6
4
2
0
-2
-4
*
*
Basal - Initial
Basal - Stimulated

LH-stimulated incubations. Furthermore, in luteal cells of Control animals incubated with LH, concentration of LH receptors tended ($P \leq .10$) to be correlated positively with the difference in concentration of free cholesterol between Basal and LH-stimulated incubations.

Discussion

Low or maintenance energy diets offered for several weeks can block estrous cyclicity of heifers (Imakawa et al., 1983; Imakawa et al., 1984). A maintenance energy diet can negatively affect luteal function long before the animals become anestrous. For example, the concentration of progesterone in serum was reduced during the first estrous cycle that heifers were offered a diet which maintained body weight rather than one that increased body weight (Imakawa et al., 1983). Thus, relative to diets which promote body weight gain, diets which only provide enough energy to maintain body weight can reduce luteal function in heifers. Since the Restricted diet maintained body weight of heifers in Experiment I, it is not clear why this diet did not significantly reduce the concentration of progesterone in serum. However, consistent with results reported regarding beef heifers (Imakawa et al., 1986b), change in body weight was correlated positively with area under the curve of progesterone. Therefore, it appears that the severity of energy restriction that a heifers experiences determines whether luteal function is reduced.

Table 7. Correlation coefficients between changes in concentration of cholesterol, luteal function and concentration of LH receptors.

Cholesterol Concentration (μg/mg protein)	Restricted Heifers			Control Heifers		
	Basal P[a]	Stim P	$LH_{R\theta}$[b]	Basal P	Stim P	$LH_{R\theta}$
Initial[c] Esterified Cholesterol (CE)	.834**	.876***	.755***	-.263	.055	.376
Initial CE - Basal CE[d]	.837**	.858***	.804**	.359	-.113	.011
Initial CE - Stim CE	.812**	.774**	.637*	-.445	-.489	-.322
Basal Free Cholesterol (FC)- Initial FC	.317	.333	.384	-.097	.687*	.400
Basal FC - Stim FC	.516	.353	.243	-.066	.902***	.735*

[a]Concentration of progesterone in cells and media (ng/10^6 cells) following incubation with 0 ng LH (Basal) and 100 ng LH (Stim) were used as measures of luteal function.

[b]Concentration of LH receptors (fMol/mg protein).

[c]Initial, before incubation; Basal, 2 h incubation with 0 ng LH; Stim, 2 h incubation with 100 ng LH.

[d]Change in concentration of cholesterol between incubations (Initial concentration minus Basal concentration).

*P≤.10

**P≤.05

***P≤.01

Restricted dietary energy intake reduced weight of CL in heifers, consistent with previous reports (Hill et al., 1970; Gombe and Hansel, 1973; Apgar et al., 1975; Harrison and Randel, 1986; Villa-Godoy et al., 1990). Independent of diet, there was a positive linear relationship between weight of CL and concentration of progesterone in serum which was similar to previous reports (Diekman et al., 1978; Spicer et al. 1981). In the current experiment the degree of energy restriction was not sufficient to reduce the concentration of progesterone in serum. However, since the Restricted diet reduced weight of CL, I suggest reduced luteal development is one mechanism by which negative energy balance may reduce concentration of progesterone in serum.

How energy balance affects the weight of CL is not clear. Increased luteal binding sites for LH has been associated positively with growth and function of CL in ewes and heifers (Diekman et al., 1978; Spicer et al., 1981) and in Control heifers in the current esperiment. However, there was no significant relationship between number of receptors for LH and weight of CL in Restricted heifers. Indeed, CL from Restricted heifers tended to weigh less but have more receptors for LH. There are at least three possible reasons for lack of association between concentration of receptors for LH and weight of CL in Restricted heifers. 1) A cause and effect relationship does not actually exist between these two variables. 2) Luteotropic compounds not quantified in this experiment such as insulin-like growth factor I or insulin may

be required for the full luteotropic action of LH to be demonstrated (May and Schomberg, 1981; O'Shaughnessy and Wathes, 1985a; Harrison and Randel, 1986; Veldhuis et al., 1986; Schams et al., 1988; Marua et al., 1988). The concentration of insulin-like growth factor I and insulin are lower during negative energy balance thus resulting in sub-optimal conditions for CL development (Harrison and Randel, 1986; Brier et al., 1988; Villa-Godoy et al., 1990). 3) Restricted energy intake decreased the concentration of LH in serum during the periovulatory period of early luteal development. Reduced concentration of LH in serum during the periovulatory period could inhibit subsequent hypertrophy and hyperplasia of luteal cells (Snook et al., 1969; Farin et al., 1985, 1986).

In previous reports negative energy balance reduced (Apgar et al., 1975) or blocked (Imakawa et al., 1983; Villa-Godoy et al., 1990) LH-stimulated production of progesterone *in vitro*. In contrast, luteal cells from heifers maintaining energy balance were as responsive to stimulation by LH as luteal cells from heifers gaining weight (Imakawa et al., 1983). In the present experiment, Restricted animals were at or near energy balance. Therefore, it is not unexpected that the LH-induced increase of progesterone in cells and media did not differ between Control and Restricted heifers.

Energy balance may affect the number of receptors for LH by affecting the composition of luteal cells in CL. Villa-Godoy et al. (1990) reported negative energy balance increased

the proportion of small luteal cells in bovine CL. Small luteal cells have a higher concentration of receptors for LH and are more responsive to stimulation by LH than are large luteal cells (Fitz et al., 1982). Increased concentration of receptors for LH in CL in Restricted heifers is consistent with increased proportion of small luteal cells in CL of energy deficient heifers. Despite increased proportion of small luteal cells however, negative energy balance reduced LH-induced luteal function *in vitro* (Villa-Godoy et al., 1990). It is possible negative energy balance reduced intracellular events (eg. availability of ATP, NADPH, C_{scc}) necessary for LH-stimulated production of progesterone and thus overcame the positive effect increased concentration of LH receptors.

Our second and third objectives were to determine the effect of energy intake on concentration and metabolism of cholesterol in luteal cells. My premise was restricted energy intake could reduce availability and uptake of LDL cholesterol and *de novo* synthesis of cholesterol in luteal cells. However, diet and change in body weight did not affect the initial concentration of cholesterol in luteal tissue. Change in body weight did affect the composition of the cholesterol within luteal tissue. Specifically, the concentration of esterified cholesterol within the CL was correlated negatively with change in body weight. Increased concentration of cholesterol esters in tissue may indicate decreased ability of luteal tissue to utilize cholesterol for production of

progesterone. For example, increased concentration of cholesterol esters in luteal tissue occurs concomitant with decreased activity of C_{scc} in bovine luteal tissue (Rodgers et al., 1986b). In addition, high concentrations of cholesterol esters are associated with decreased luteal function in cows (Hafs and Armstrong, 1968) and tended to be correlated negatively with concentrations of progesterone in serum in the current experiment. Thus, increased concentration of esterified cholesterol in animals losing weight may be an important indicator of compromised luteal function *in vivo*.

Alternatively, increased initial concentration of cholesterol esters in CL from animals losing body weight may also reflect a change in the luteal cell population. Though not examined in this experiment, increased number of small luteal cells could increase concentration of esterified cholesterol in luteal cells of animals losing weight. The cytoplasmic volume occupied by lipid vesicles is larger in small than in large cells (Priedkalns and Weber, 1968a). The majority of cholesterol in the cytoplasm is esterified to fatty acids (Strauss et al., 1981). Therefore, increased concentration of cholesterol esters in luteal cells of Restricted heifers is consistent with an increased proportion of small luteal cells.

In luteal cells of Restricted heifers there was a positive relationship between initial concentration of cholesterol esters and concentration of progesterone following incubation. Apparently cholesterol esters were used for

synthesis of progesterone during incubation. Use of esterified cholesterol in steroidogenesis in luteal tissue of ruminants has been reported previously. The activity of cholesterol esterase in luteal tissue is stimulated by LH and is correlated positively with concentration of progesterone in serum during diestrus in ewes (Caffrey et al., 1979). Similarly, the concentration of cholesterol esters is lowest in luteal tissue of heifers during maximal production of progesterone (Hafs and Armstrong, 1968). Thus, cholesterol esters may be used in luteal steroidogenesis by growing heifers as well as in Restricted fed animals. There was no evidence that Restricted energy intake impaired mobilization of cholesterol esters or use of cholesterol for steroidogenesis in luteal cells.

Concentration of cholesterol increased during the 2 h incubation in luteal cells of Control but not Restricted heifers. Since the production of progesterone by luteal cells did not differ between treatment groups, it appears that restricted energy intake reduced *de novo* synthesis of cholesterol in luteal cells. Lange et al. (1988) reported that newly synthesized cholesterol is used preferentially over esterified cholesterol in steroidogenesis. Thus, reduced ability to synthesize cholesterol may ultimately interfere with optimal steroidogenesis.

The mechanism by which energy balance affected synthesis of cholesterol can not be determined. However, restricted energy intake may reduce activity of HMG-CoA reductase [the

enzyme which catalyzes the rate limiting step in synthesis of cholesterol (Luskey, 1988)]. In addition, restricted energy intake may reduce availabilty of precursors for synthesis of cholesterol. Negative energy balance reduced activity of HMG-CoA reductase in liver of rats (Dietschy and Brown, 1974). Insulin is responsible for the stimulatory effect of positive energy balance on activity of HMG-CoA reductase activity in liver (Zammit and Easom, 1987). As previously mentioned, insulin can stimulate production of progesterone in luteal tissue. The effect of energy balance and insulin on the activity of HMG-CoA reductase in bovine luteal tissue is not known, however. Acetate and glucose are used in production of progesterone by bovine luteal tissue (Savard and Casey, 1964; Savard et al., 1965; Armstrong and Black, 1966, 1968; Armstrong et al., 1970; Pate and Condon, 1989). Concentration of acetate (Lomax and Baird, 1983) and glucose (de Boer et al., 1985; McCann and Hansel, 1986) in blood are reduced during restricted energy intake in cattle. Whether reduced concentration of acetate and glucose in blood during negative energy balance affects luteal synthesis of cholesterol has not been examined.

Heifers maintaining body weight represent an intermediate step between positive to negative energy balance. I believe changes in luteal function noted in heifers in maintaining body weight are indicative of changes which eventually impair luteal function of animals in negative energy balance. In the present study, we found no evidence that restricted energy

intake reduced the concentration of receptors for LH on luteal tissue. There was evidence that restricted energy intake may actually increase receptors for LH on luteal cells. However, restriced energy intake reduced the amount of cholesterol synthesized within CL. In addition, in animals losing body weight concentration of esterified cholesterol in luteal tissue increased and may indicate reduced ability of CL to mobilize cholesterol esters for steroidogensis. Whether the effects of restricted energy intake on luteal cholesterol metabolism are exacerbated and result in reduced luteal function in animals during negative energy balance remains to be tested. In addition, it seems likely that reduced concentration of progesterone in serum of animals during negative energy balance results from reduced weight of CL.

EXPERIMENT II: INFLUENCE OF NEGATIVE ENERGY BALANCE ON HMG-CoA REDUCTASE ACTIVITY, AND CHOLESTEROL METABOLISM IN BOVINE LUTEAL TISSUE.

Introduction

In Experiment I, CL tended to weigh less in heifers maintaining body weight compared with heifers gaining body weight. However, diets that maintained body weight did not affect basal or LH-stimulated luteal function *in vitro* (Imakawa et al., 1983; Experiment I). Diets which cause negative energy balance reduced weight of CL, reduced the concentration of progesterone in serum, and reduced or blocked the ability of LH to stimulate luteal tissue (Apgar et al., 1974; Imakawa et al., 1983; Villa-Godoy et al., 1990). Thus, in heifers maintaing body weight the magnitude of luteal function is intermediate between animals in positive and negative energy balance. Indeed, the concentration of progesterone in serum is correlated positively with change in body weight of heifers (Imakawa et al., 1986b; Experiment I). Compared with gaining body weight, maintaining or losing body weight increased concentration of esterified cholesterol and reduced endogenous synthesis of cholesterol in bovine CL (Experiment I). I suggested that negative energy balance would exacerbate these changes in cholesterol metabolism, and I suggested that these changes would eventually reduce luteal

function. Thus, one goal of Experiment II was to test these hypotheses.

Reduction in synthesis of cholesterol by luteal cells may decrease production of progesterone. Thus, the mechanism by which energy balance affects synthesis of cholesterol in luteal cells is of interest. Energy balance could affect *de novo* synthesis of cholesterol in luteal cells by affecting: 1) the activity of HMG-CoA reductase, 2) the conversion of cholesterol into progesterone [reduced conversion of cholesterol to steroids reduces the *de novo* production of cholesterol by feedback inhibition (Rodgers et al., 1987a; Luskey 1988)], or 3) the concentration of metabolites which are used in cholesterol synthesis in luteal cells.

The objectives of the second experiment were to determine the effect of negative energy balance on the activity of HMG-CoA reductase and conversion of radiolabelled cholesterol into progesterone in bovine luteal cells. An additional objective was to determine the effect of metabolites for *de novo* cholesterol synthesis on production of progesterone in luteal cells from animals in positive and negative energy balance.

Materials and Methods

Materials

Materials were the same as for Experiment I with the following exceptions and additions. Minimum essential media with nonessential amino acids and .25 mg/ml glucose (MEM), Specialty Media, Inc. (Lavallette, NJ); R-(-)-3-

hydroxybutyric acid, sodium salt, Aldrich Chemical Company, Inc. (Milwaukee, WI); acetonitrile (Chrom AR), ethyl ether, Mallinkrodt (Paris, KY); sodium acetate, acetoacetic acid lithium, a-D[+]-glucose, Sigma (St. Louis, MO); [1,2,6,7-^{3}H]cholesterol, 93.8 Ci/mMol, Dupont (Wilmington, DE); {1,2,6,7-^{3}H]progesterone, 85 Ci/mMol, Amersham (Arlington Heights, IL); NIH-LH B4, National Institute of Arthritis, Metabolism and Digestive Diseases (Bethesda, MD); soda-lime scintillation vials, Safety-Solve Counting Cocktail, Research Products International Corporation (Mount Prospect, IL).

Animals

Estrous cycles of 12 Holstein heifers were synchronized with two injections of PGF spaced 11 days apart. To determine the day of estrus, heifers were observed for estrous behavior three times daily. Estrus was defined as the day a heifer stood to be mounted (day 0). Heifers were assigned randomly to one of two diets (Table 8) to begin on day 0 of the first synchronized estrous cycle. The Control diet was designed to promote body weight gain (LWG) of .6 kg/day (NEM + NEG). The Restricted diet was designed to provide energy intake below maintenance requirements (70% NEM). NRC formulas (NRC, 1988) were used to determine the amount of energy that was required in diets to achieve these goals (NEM= $.086BW^{\cdot 75}kg$; NEG=$(.045BW^{\cdot 75}kg)(LWGx1.119)$ + LWG). Otherwise, diets met NRC recommendations for protein, vitamins and minerals for maintenance plus growth (Control diet) or for maintenance (Restricted diet). To control and monitor dietary intake,

Table 8. Composition of Diets[a]

Item	Treatment Group	
	Control	Restricted
Ingredients, %		
Corn Silage	86.0	None
Protein, Vitamin, Mineral Mix[b]	14.0	43.0
Alfalfa Hay, ground	–[c]	57.0
Dry Matter Intake (kg/day/325 kg heifer)	17.8	6.6
Energy Balance (NEM[d], MCal/day/325 kg heifer)	+3.4	-2.0

[a]Dry matter basis.

[b]Protein, mineral, vitamin mix composition: soybean meal, 91.4%; calcium sulfate, .87%; copper sulfate, .02%; dicalcium phosphate, 2.96%; ferrous sulfate, .09%; limestone, .28%; manganese oxide, .01%; magnesium oxide, .76%; potassium chloride, .15%; selenium 200 ppm, .95%; trace mineral salt, 1.88%; 13.9 KIU vitamin A/kg; 1.98 KIU vitamin D/kg; 256 IU vitamin E/kg.

[c]In addition to diet fed at 0600h, Control heifers received .8 kg ground alfalfa hay at 1800 daily.

[d]Net energy for maintenance.

heifers were moved to an indoor facility, housed and fed in individual pens beginning on day 0 or day 1 postestrus of the first synchronized estrous cycle. Orts were weighed and discarded daily and new feed was offered at 0900. Body weight of heifers was determined on two consecutive days each week. Change in body weight each week was used to adjust the total amount of feed offered each heifer. To monitor length of estrous cycles, heifers were moved to a group pen twice daily for 30 min, and observed for estrous behavior. There was no access to feed or water during these periods. To monitor luteal function *in vivo*, jugular blood was sampled at 0600 daily and samples were allowed to clot. After centrifugation serum was collected and stored at -20°C for determination of concentration of progesterone.

Tissue

On day 10, 11 or 12 (10.7 ± .2, SEM) of the second estrous cycle following synchronization, heifers were tranquilized with xylazine hydrochloride (1 mg/100 lb BW, i.v.). Upon collection per vaginum, CL were rinsed and then stored in HBSS and transported to the lab as described for experiment I. After adherent connective tissue was removed, CL were weighed, quartered and .5 mm slices were obtained using a Stadie Riggs hand microtome. Slices weighing approximately 500 mg were placed in 20% glycerol MEM (vol:vol)and stored at -70°C for determination of total number of receptors for LH per CL (Spicer et al., 1981). Slices weighing 200 to 400 mg were placed in cryovials and stored in

liquid nitrogen for determination of HMG Co-A reductase activity. The remaining tissue slices were dispersed enzymatically as described previously (Villa-Godoy et al., 1990). The final preparation of dispersed luteal cells was diluted with MEM (250,000 cells/ml). Aliquots of cells or cells and media were collected to determine initial concentration of progesterone, cholesterol, protein (Lowry et al., 1951) and receptors for LH in luteal cells as described in Experiment I.

Luteal Function *In Vitro*

For analyses of luteal function and cholesterol metabolism, glucose was added to MEM to a final concentration of 1 mg/ml. Cells were incubated for 2 hours at 37°C in a shaking water bath with 0 ng (Basal) or 100 ng (Stimulated) LH. Following incubation, test tubes were centrifuged (10 min at 800 x g). Media and cells were then placed in separate polypropylene tubes, frozen and stored at -20°C until assayed for progesterone or cholesterol.

To determine the effect of negative energy balance on ability of luteal cells to convert cholesterol into progesterone, cells were incubated with 1.4 µCi ^{3}H-cholesterol in 20 µl ethanol of ethanol alone in MEM. After 2 h, cells and media were separated and stored as described above. To determine the amount of radioactivity from ^{3}H-cholesterol that was incorporated into steroids, extracts from cells and media incubated with ^{3}H-cholesterol were subjected to high performance liquid chromatography (HPLC). Counts of

radioactivity which co-eluted with progesterone during HPLC was defined as incorporation of ^{3}H-cholesterol label into progesterone. Specific activity of progesterone which was produced during incubation was also determined. Specific activity was defined as the radioactivity which co-eluted with progesterone during HPLC/production of progesterone during 2 h incubation with 20 µl ethanol.

To determine the effect of metabolites for cholesterol synthesis on production of progesterone, cells were incubated in .25 mg/ml glucose MEM. Metabolites were added separately to achieve final concentration as listed: acetate (0, .05, .10 mg/ml); glucose (.25, .50, 1.00 mg/ml); acetoacetate and 3-hydroxybutyrate (1:10, wt:wt; 0, .02, .10 mg/ml). After 2 h, cells with media were frozen and stored at -20°C until analyzed for concentration of progesterone.

Radioimmunoassay of Progesterone

Concentration of progesterone was determined in serum, media and cells as described previously (Spicer et al., 1981, as modified for cells in Experiment 1). To determine the within and between assay coefficients of variation, pooled samples of cells, media or serum with low and high concentration of progesterone were included in assays. Coefficients of variation of samples with a high concentration of progesterone averaged 4.9% within and 10.5% among assays. Coefficients of variation of samples with a low concentration of progesterone averaged 5.2% within and 11.8% among assays.

LH Binding Assay

Human chorionic gonadotropin (hCG, CR-121; 13,450 IU/mg) was radioiodinated as described previously (Spicer et al., 1981). Specific activity of ^{125}I-hCG was 113 cpm/pg and maximum specific binding to fresh luteal homogenates was 43%. Number of binding sites in luteal cells were determined by Scatchard plots constructed from four point saturation curves using 2.5 x 10^5 luteal cells as described in Experiment I. Number of binding sites per CL were determined by Scatchard plots constructed from four point saturation curves using 2.5 mg luteal homogenates as described by Spicer et al. (1981).

Concentration of Cholesterol in Luteal Cells

Concentration of total cholesterol, unesterified cholesterol, and esterified cholesterol were determined in pellets of 2.5 x 10^5 luteal cells as described in Experiment I. Intraassay coefficients of variation were 6.5 and 6.8% and interassay coefficients of variation were 2.5 and 8.9% for total and free cholesterol, respectively.

HMG-CoA Reductase Activity

Cryovials containing luteal slices were transferred from liquid nitrogen and shipped on dry ice to Dr. Salman Azhar (Veterans Administration Medical Center, Palo Alto CA). Dr. Azhar determined the total activity of HMG-CoA reductase in the microsomal fraction of luteal tissue (Azhar et al., 1984). Data were expressed as picomole [^{14}C]-mevalonic acid produced$\cdot$ $min^{-1}\cdot mg^{-1}$ microsomal protein.

HPLC of Incubated Luteal Cells

Cells and media incubated with ^{3}H-cholesterol were extracted 3 times with 10 ml ether. Following centrifugation (2 min, 250 x g), the aqueous phase was frozen and the supernatant was collected, dried under nitrogen, and resuspended in 200 µl acetonitrile. For separation of compounds, 150 µl of the reconstituted sample was injected onto a 3.9 x 150 mm C-18 column held at 50°C (µBondapak; Millipore Corporation, Waters Chromatography Division, Milford, MA). The sample was eluted with a gradient flow as follows: 0 to 3.6 min, 30% acetonitrile; 3.6 to 15.6 min, 80% acetonitrile; 17 to 35 min, 100% acetonitrile. Flow rate was 2 ml/min and fractions were collected into scintillation vials at one minute intervals for 35 min, except for fractions that co-eluted with progesterone (4:30-5:30; min:sec), cholesterol (13:45-16:00), and cholesteryl oleate (28:30-30:30) standards. The resulting 33 fractions were dried, resuspended in 6 ml counting cocktail (counting efficiency of cocktail= 54.7%) and counted (Isocap 300, TM Analytic, Elk Grove Village, IL; counting efficiency of counter = 53.1%). Radioactive counts that are reported in fractions from samples were corrected for efficiency of recovery of cpm added to incubations (93% ± 2.33%, SEM). In addition, cpm in fractions were corrected for impurities in the ^{3}H-cholesterol preparation used in incubations (as described in Appendix 2). Validation of separation of cholesterol and progesterone by HPLC is described in Appendix 2.

Statistical Analyses

To describe luteal function *in vivo*, area under the profile of progesterone in serum was calculated for all of estrous cycle 1 and for day 1 to day 10 postestrus of estrous cycle 1 and 2. Split plot analysis of variance with estrous cycle as the subplot was used to examine the effect of diet on the profile of progesterone from day 1 to day 10 of estrous cycle 1 and 2. One way analysis of variance was used to examine the effect of diet on body weight, weight of CL, length of estrous cycle, number and affinity of receptors for LH, activity of HMG-CoA reductase, and area under the profile of progesterone in serum. Split plot analysis of variance with incubation (Initial, Basal, and Stimulated) as the subplot was used to examine the effect of diet or presence of LH on concentrations of cholesterol and progesterone in dispersed luteal cells and/or media. When a significant main effect or interaction was detected specific contrasts were examined with Student's t tests (Gill, 1986). Split-split plot analysis of variance (Gill, 1988) was used to determine main effects and interactions of diet, concentration of metabolite, and concentration of LH on production of progesterone. Production of progesterone was defined as the concentration of progesterone in samples following incubation minus the concentration of progesterone before incubation (Initial). When a significant main effect of metabolite was detected, Dunnet's test was used to examine differences between the lowest and higher concentrations of a particular

metabolite. *A posteriori* contrasts were examined with Scheffe's test (Gill, 1986).

It became obvious during analysis of samples, that the preparation of ^{3}H-cholesterol used was not homogeneous. Specifically, during HPLC, cpm from ^{3}H-cholesterol did not elute in one peak (See Appendix II, Figure 15 for details). Thus it became necessary to determine if cpm in fractions of samples were due to impurities in ^{3}H-cholesterol, or due to incorporation of ^{3}H-cholesterol into different compounds. To accomplish this, HPLC data from samples were compared with data from ^{3}H-cholesterol alone. Counts in each fraction were expressed as a % of total cpm recovered in all fractions. Percent of total cpm in fractions collected from samples were contrasted with percent of total cpm in fractions collected from 20µl ^{3}H-cholesterol. Differences between samples and ^{3}H-cholesterol were examined with Dunnet's test. When there was a significant difference ($P \leq .05$) between cpm from samples and cpm from ^{3}H-cholesterol, data were analyzed further. First, cpm due to impurities in ^{3}H-cholesterol were subtracted from cpm in fractions of cell and media extracts. Data which were corrected for background were then used to examine effect of diet on incorporation of ^{3}H-cholesterol into compounds eluting in fractions. These data were analyzed with split plot analysis of variance with fraction as the subplot.

Some radioactivity co-eluted with compounds which could not be identified (Appendix II). Level of absorbance can be used to estimate the concentration of compounds eluting during

HPLC. It was of interest to determine if diet affected the concentration of these unidentified compounds. To accomplish this, the absorbance profiles for samples of cells and media were corrected for extraction efficiency (cpm recovered/cpm added) and number of cells in the original sample. Split plot analysis with elution time as the subplot was then used to examine if diet affected the profile of absorbance of samples.

Analyses were performed with mainframe SAS (1985b) using the GLM option for unbalanced data. In some cases Pearson correlation coefficients were determined between variables of interest using the CORR option of SAS (1985a).

Results

Animals

One heifer was not observed in estrus after being placed on the Restricted diet. This heifer lost 37 kg between day 0 and day 21 of the experiment. Based on concentration of progesterone in serum, this animal demonstrated a normal luteal phase following the first synchronized estrus. However, following luteal regression, this heifer became anovulatory. The concentration of progesterone remained $\leq$ 0.2 ng/ml for 18 days, there were no palpable luteal structures on either ovary, and no detected estrous behavior in this heifer. Data from this animal were not included in analyses. Thus, there were six heifers in the Control group and five in the Restricted group.

Initial body weights did not differ between heifers

assigned to the two diets and averaged 336 ± 2.9 kg (± SEM). Body weight of Control heifers increased (P≤.01), whereas Restricted heifers lost body weight (P≤.01) during the experiment (+19.33 versus -33.80 ± 5.03 kg, SEM). Average daily change in body weight was +.67 kg in Control heifers, and -1.068 in Restricted heifers (±.175, SEM).

Diet did not affect the length of estrous cycle 1 (20.3 ± 1.16 days, ± SEM) or the profile of progesterone in serum during estrous cycle 1 (Table 9; P=.15). However, diet and estrous cycle number interacted to affect the profile of progesterone in serum during the first 10 days of estrous cycle 1 and cycle 2 (P≤.05). The profile of progesterone in serum during estrous cycle 2 tended to be higher in Control heifers than in Restricted heifers (P≤.10). In addition, the trend in the profile of progesterone in serum between estrous cycle 1 and estrous cycle 2 increased in Control heifers but decreased in Restricted heifers (P≤.05).

Binding Sites for LH

Affinity of binding sites for LH did not differ between treatment groups (Table 10). Concentration of binding sites for LH in luteal cells of Restricted hiefers was higher than in cells from Control heifers (P≤.02). But, total number of binding sites for LH per CL tended to be higher in Control heifers than in Restricted heifers (P≤.10). This is probably because luteal weight in Control heifers was greater (P≤.01) than in Restricted heifers (Table 9).

Table 9. Effect of Diet on Concentration of Progesterone in Serum and Weight of CL.

Treatment Group	Concentration of Progesterone in Serum[a]			
	Estrous Cycle 1 (Entire Cycle)[b]	Estrous Cycle 1 (Day 1 to Day 10)[c]	Estrous Cycle 2 (Day 1 to Day 10)	CL Weight[d] (gm)
Control	35.5	11.68	13.50	5.91
Restricted	51.0	14.91	9.81	3.06

[a]Data are expressed as area under the profile of progesterone in serum ($ng \cdot ml^{-1} \cdot day$).

[b]Within the column, SEM = ± 7.67.

[c]For area under the profile of progesterone in serum between day 1 to day 10 in estrous cycle 1 and 2 there was a treatment by time interaction ($P \leq .05$). During cycle 2, area under the profile of progesterone tended ($P \leq .10$) to be less in Restricted animals than in Controls. In addition diet affected the trend in area under the profile of progesterone between cycle 1 and cycle 2 ($P \leq .05$). Pooled SEM during cycle 1 and 2 = ± 1.586.

[d] Means within the column differ ($P \leq .01$), SEM = ± .598.

Table 10. Effect of Diet on Affinity Constants (K_a) and Numbers of Unoccupied Binding Sites for ^{125}I-hCG on Dispersed Luteal Cells or in Homogenates of Luteal Tissue.

Treatment Group	Affinity Constant[a] ($x10^{10}M^{-1}$)	Number of Binding Sites	
		Dispersed Luteal Cells[b] (fMol/mg protein)	CL[c] (pMol/CL)
Control	1.99	22.09	6.77
Restricted	2.42	39.49	4.39

[a]SEM = ±.298.

[b]Means within the column differ (P≤.02), SEM = ± 4.30.

[c]Number of binding sites per CL were estimated by determining the concentration of receptors per mg protein in luteal homogenates. The concentration of receptors per mg protein was then multiplied by the concentration of protein per mg luteal homogenate and weight of CL. Within the column SEM = ± .96.

Steroidogenic Function of Luteal Cells

There was no effect of diet or diet by incubation (Initial, Basal, Stimulated) interaction on concentration of progesterone in luteal cells or media. Data from the two treatment groups were therefore combined (Figure 6). There was a main effect of incubation ($P \leq .0001$) on concentration of progesterone. During the 2 h incubation with 0 ng LH (Basal), concentration of progesterone in media and cells increased 36%. Addition of LH (Stimulated) did not further increase the production of progesterone by incubated cells. It was of interest to determine if the lack of stimulation by LH on concentration of progesterone was due to the preparation of LH, or due to problems with the incubation. To determine if the preparation of LH was a problem, I tested the ability of LH from Experiment I and Experiment II to compete for binding with ^{125}I-hCG on luteal cells in a radioreceptor assay. The preparation of LH from Experiment I, depressed binding of ^{125}I-hCG on luteal cells by 78%. However, the preparation of LH from Experiment II depressed binding of ^{125}I-hCG on luteal cells by only 22%. As the preparations of LH were prepared the same way in both experiments, it appeared the LH used in Experiment II was less potent than that used in Experiment I. Since LH did not cause a significant increase in production of progesterone, data from cultures receiving LH were excluded from other analyses.

There was no effect of diet or diet by incubation interaction on concentration of free cholesterol (Figure 7).

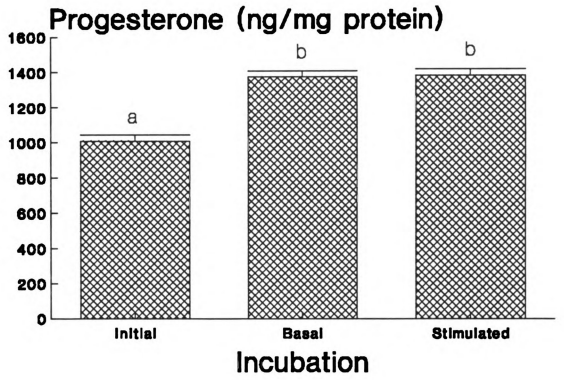


Figure 6. Concentration of progesterone in luteal cells and media before incubation (Initial) and following a 2 h incubation with 0 ng LH (Basal) or with 100 ng LH (Stimulated). There was no significant effect of diet or diet by incubation interaction, thus data represent pooled means (+SEM). [ab]Bars with different superscripts differ ($P \leq .001$).

However, incubation affected the concentration of free cholesterol ($P \leq .002$). In both treatment groups, concentration of free cholesterol increased during the 2 h incubation ($P \leq .05$).

Diet and incubation interacted ($P \leq .001$) to affect concentration of esterified cholesterol in luteal cells. Initial concentration of esterified cholesterol in luteal cells of Restricted heifers was 3.5 fold greater ($P \leq .02$) than in luteal cells of Control heifers. The concentration of cholesterol esters increased during incubation of luteal cells from Control heifers ($P \leq .05$) and decreased during incubation of luteal cells of Restricted heifers ($P \leq .01$). Since diet affected the concentration of esterified cholesterol in luteal cells as was predicted, it was of interest to determine if the concentration of esterified cholesterol was related to other aspects of luteal function in Restricted animals. In Restricted animals, the initial concentration of esterified cholesterol esters was correlated negatively with the initial concentration of progesterone in luteal cells ($r=-.88$, $P \leq .05$), and with the concentration of progesterone in media and cells following the 2 h incubation ($r=-.94$, $P \leq .02$).

Diet and incubation interacted ($P \leq .07$) to affect the concentration of total cholesterol in luteal cells. The initial concentration of total cholesterol tended ($P \leq .10$) to be higher in luteal cells of Restricted than in Control animals. However, concentration of free and esterified cholesterol increased during incubation in luteal cells of

Control heifers. As a result, the concentration of total cholesterol increased during incubation in luteal cells of Control heifers ($P \leq .001$). In contrast, concentration of free cholesterol increased and concentration of esterified cholesterol decreased during incubation so there was no net change in concentration of total cholesterol in luteal cells from Restricted heifers during incubation. Thus, there was net synthesis of cholesterol during the incubation in luteal cells of Control heifers, but not in luteal cells of Restricted heifers.

HMG-CoA Reductase Activity

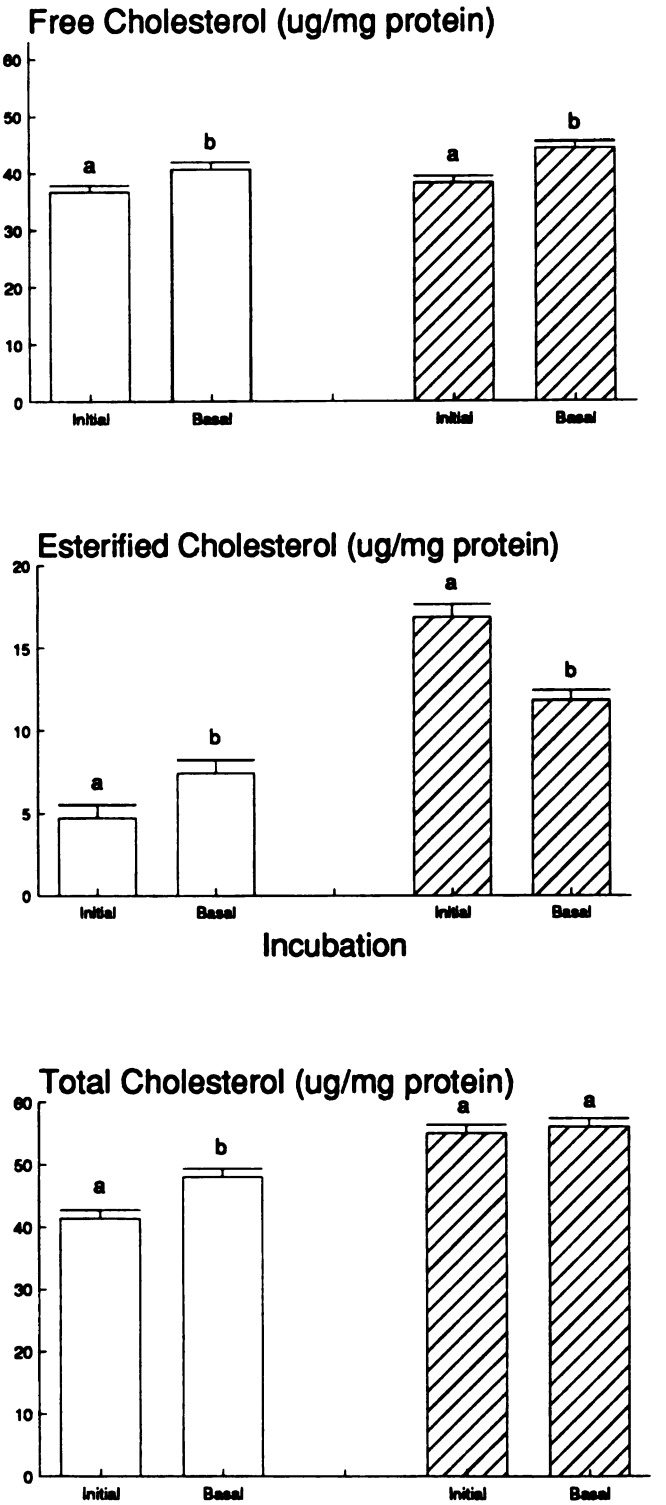
Diet did not affect activity of HMG-CoA reductase in luteal tissue. Activity of HMG-CoA reductase (nMol mevalonic acid produced$\cdot min^{-1} \cdot mg^{-1}$ microsomal protein) in luteal cells averaged 2.75 in Control heifers and 3.09 in Restricted heifers (± .26, SEM). Activity of HMG-CoA reductase was not correlated with concentration of progesterone in serum, or concentration of progesterone in media or cells after incubation.

Incorporation of Labelled Cholesterol into Progesterone

Fractions 6 to 12 from cells and fractions 21 to 33 for both cells and media had significantly higher cpm than did background cpm from ^{3}H-cholesterol standard (Figure 8). There were several fractions which contained cpm that did not co-elute with standards tested (Figure 8, A and B; Appendix II) and demonstrated significant absorbance (Figure 8, C and D). Thus, these fractions contain unidentified compounds. Diet

Figure 7. Effect of diet on concentration of free (unesterified), esterified, and total (esterified and unesterified) and cholesterol in luteal cells. Values represent means (+ SEM) of Initial concentration of cholesterol and concentration of cholesterol following a 2 h incubation with 0 ng LH (Basal, 2 h). Control animals are represented by open bars, Restricted animals are represented by hatched bars. Between treatment groups, the Initial concentration of esterified cholesterol was higher in luteal cells of Restricted animals ($P \leq .02$). ^{ab}Within treatment group and type of cholesterol, bars with different superscripts differ ($P \leq .05$).

Figure 7.


Free Cholesterol (ug/mg protein)
60
50
40
30
20
10
0
a
b
a
b
Initial
Basal
Initial
Basal
Esterified Cholesterol (ug/mg protein)
20
15
10
5
0
a
b
a
b
Initial
Basal
Initial
Basal
Incubation
Total Cholesterol (ug/mg protein)
60
50
40
30
20
10
0
a
b
a
a
Initial
Basal
Initial
Basal

did not affect radioactivity which coeluted with these unidentified compounds or the profiles of absorbance at 194 and 242 nm. Thus, the absorbance profiles of an extract of cells (C) and media (D) of only one heifer (Control, animal 3) is shown for illustration.

In fractions collected from media, diet did not affect the amount of radioactivity in fractions. In fractions from samples of cells, there was a significant interaction between diet and fraction number. Controls had more ($P<.05$) radioactivity in fraction 6 (co-eluting with progesterone standard) and a tendency ($P\leq.10$) for more radioactivity in fraction 30 (co-eluting with cholesteryl oleate standard) than did samples from Restricted heifers (Table 11). Diet did not affect the specific activity of progesterone produced in luteal cells during incubation (Table 11).

Addition of Metabolites to .25 mg/ml Glucose MEM

There was no significant effect of diet or interaction between diet and concentration of acetate or glucose on production of progesterone by bovine luteal cells. Concentration of acetate and glucose in media affected production of progesterone by luteal cells. Relative to cultures receiving .05 mg/ml acetate, .10 mg/ml acetate increased production of progesterone by luteal cells ($P\leq.05$). Relative to cultures receiving .50 mg/ml glucose, 1.00 mg/ml glucose tended to increase production of progesterone by luteal cells ($P\leq..10$).

There was an interaction between diet and concentration

Figure 8. Effect of diet on the HPLC elution profile of extracted cells or media. Background counts from ^{3}H-cholesterol were subtracted from radioactivity reported in fractions from cells (A) or media (B). Time that the fraction was collected is indicated on the x axis. Arrows at retention times for progesterone, cholesterol and cholesteryl oleate are included as markers for these compounds. Points represent means of Control (-*-) or Restricted (-▲-) animals (+ or - SE). Only points with cpm significantly ($P \leq .05$) above background cpm from ^{3}H-cholesterol are graphed. Absorption profiles of an extract of cells (C) or media (D) at 194 and 242 nm of one heifer (Control, Animal 3) are displayed for comparison.

Figure 8.

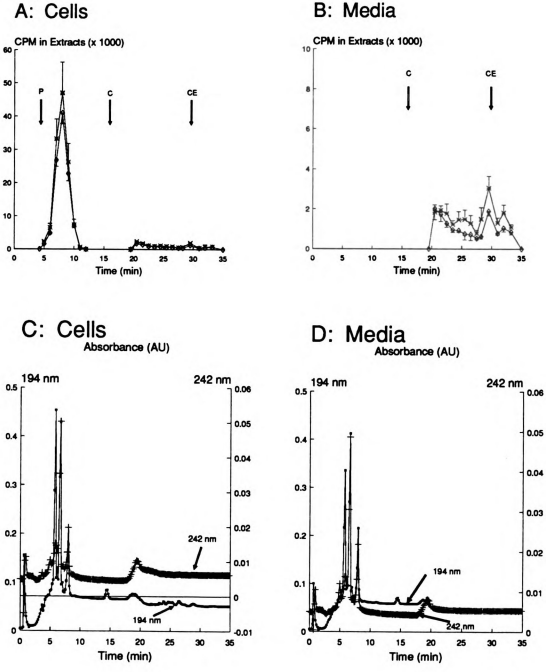

A: Cells
CPM in Extracts (x 1000)
P
C
CE
Time (min)
B: Media
CPM in Extracts (x 1000)
C
CE
Time (min)
C: Cells
Absorbance (AU)
194 nm
242 nm
242 nm
194 nm
Time (min)
D: Media
Absorbance (AU)
194 nm
242 nm
194 nm
242 nm
Time (min)

Table 11. Effect of Diet on Incorporation of ^{3}H-Cholesterol into Progesterone and Cholesteryl Oleate

Treatment Group	Incorporated Radioactivity (cpm/10^{6} cells)		Specific Activity of Progesterone Formed During Incubation[d] (cpm/ng)
	Progesterone[a] (Fraction 6)	Cholesterol Oleate[b] (Fraction 30)	
Control	2307 ± 282[c]	1981 ± 288	36.1 ± 5.2
Restricted	1546 ± 160	1352 ± 78	24.5 ± 5.2

[a]Means within the column differ ($P \leq .05$).

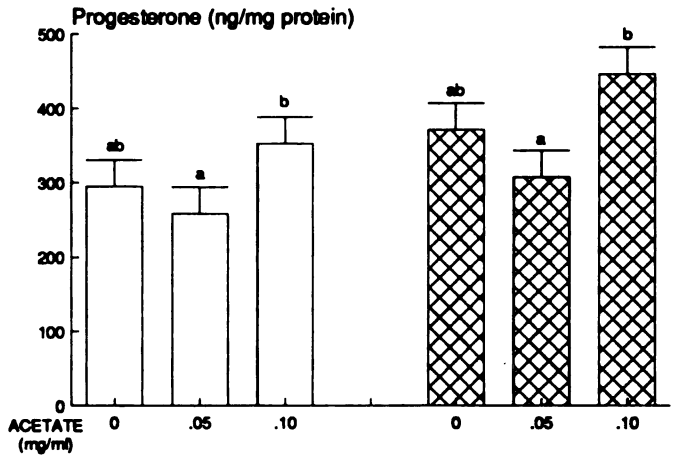
[b]Means within the column tend to differ ($P \leq .10$).

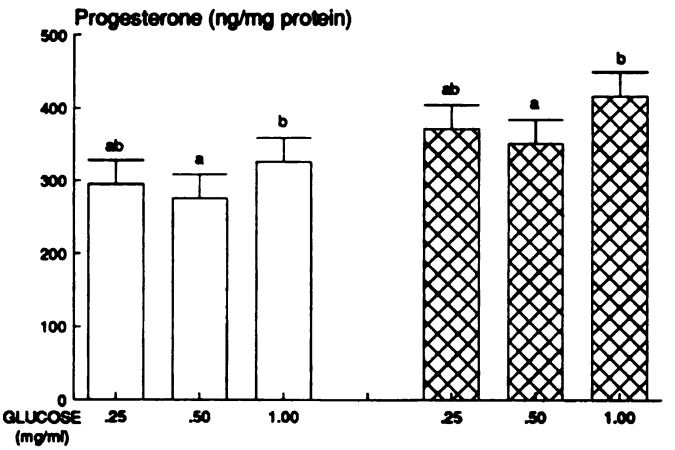
[c]Data are expressed as treatment means (± SE).

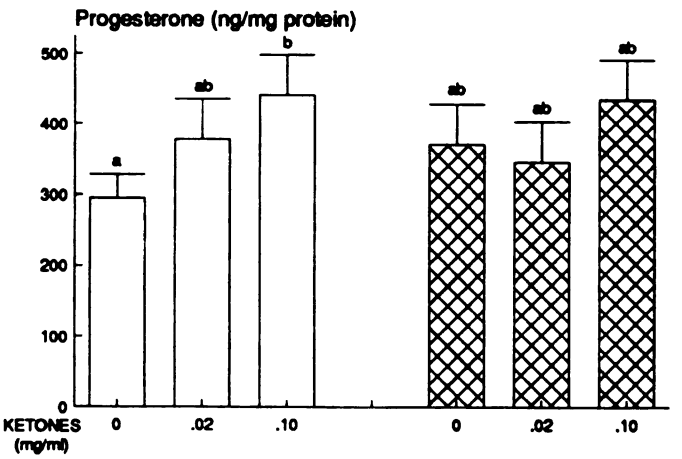
[d]Means within the column do not differ ($P \leq .18$)

Figure 9. Effect of diet and concentration of acetate, glucose and ketones on production of progesterone following a 2 h incubation with 0 ng LH. Ketones are a mixture of acetoacetate and 3-hydroxybutyrate (1:10, wt:wt). Data from Control animals are represented by open bars, data from Restricted animals are represented by hatched bars. Concentration of progesterone in cells prior to incubation have been subtracted. Data are expressed as pooled means (+SED). ^{a,b}Within acetate and ketones, means with no common superscripts vary ($P \leq .05$). Within glucose, means with no common superscripts tend to differ ($P \leq .10$).

Figure 9.


Progesterone (ng/mg protein)
500
400
300
200
100
0
ab
a
b
ab
a
b
ACETATE (mg/ml)
0
.05
.10
0
.05
.10


Progesterone (ng/mg protein)
500
400
300
200
100
0
ab
a
b
ab
a
b
GLUCOSE (mg/ml)
.25
.50
1.00
.25
.50
1.00


Progesterone (ng/mg protein)
500
400
300
200
100
0
a
ab
b
ab
ab
ab
KETONES (mg/ml)
0
.02
.10
0
.02
.10

of ketones (acetoacetate and 3-hydroxybutyrate; 1:10, wt:wt) on the production of progesterone by bovine luteal cells. In luteal cells of Control heifers addition of .10 mg/ml ketones increased production of progesterone relative to when no ketones were present ($P \leq .05$; Figure 9). However, in luteal cells of Restricted animals dose of ketones did not affect production of progesterone.

Discussion

The Restricted diet reduced body weight and caused negative energy balance in heifers. Therefore, we could examine the effect of negative energy balance on luteal development and function, concentration of LH receptors and metabolism of cholesterol in bovine luteal cells. Consistent with results in previous studies (Hill et al., 1970; Gombe and Hansel, 1973; Apgar et al., 1975; Harrison and Randel, 1986; Villa-Godoy et al., 1990), compared with CL of heifers gaining body weight, loss of body weight profoundly decreased (50%) weight of CL in heifers. However, the Restricted diet reduced concentration of progesterone in serum only 27% during the second estrous cycle. Thus, the magnitude of reduction in concentration of progesterone in serum was less than the magnitude of reduction in weight of CL in Restricted heifers.

It is not clear why the magnitude of reduction in weight of CL was greater than the magnitude in reduction in the concentration of progesterone in serum in Restricted animals. It is possible that negative energy balance may have increased

total blood volume or decreased the clearance rate of progesterone in blood which could increase concentration of progesterone in blood. Another possible explanation is that the concentration of receptors for LH on luteal cells is increased in Restricted heifers compared with Control heifers and increased concentration of receptors for LH offset the negative effect of reduced luteal weight. LH is the primary luteotropin in cattle (Hansel et al., 1973; Hoffman et al., 1974); Fairchild and Pate, 1987; Poff et al., 1988). Concentration of receptors for LH on CL are correlated positively with concentration of progesterone in cattle (Spicer et al., 1981; Garverick et al., 1985). In Experiment I there was no negative effect of short term restriction on LH-stimulated luteal response *in vitro*. Response of luteal tissue to LH-stimulation *in vivo* is also a function of concentration of LH in blood. Though not determined in the current experiment, concentration of LH in blood during the luteal phase was not reduced by negative energy balance in cattle in previous studies (Harrison and Randel, 1986; Imakawa et al., 1986b; Villa-Godoy et al., 1990). Thus, during short term negative energy balance, increased concentration of receptors for LH in luteal cells may, in part, offset the negative effect of reduced weight of CL on concentration of progesterone in serum.

In contrast to short term energy restriction in Experiment 1 and 2, long term negative energy balance reduced or blocked LH-stimulated luteal function (Imakawa et al.,

1986b; Villa-Godoy et al., 1990). Thus, the positive effect of negative energy balance might have on concentration of receptors for LH would not result in improved luteal function during long term energy balance.

Initial concentration of esterified cholesterol was highest in luteal cells from Restricted heifers. Cholesterol stored esterified to fatty acids in bovine luteal cells may normally contribute to production of progesterone. Cholesterol ester hydrolase liberates cholesterol from fatty acid esters. Luteotropic compounds, including LH and cAMP, stimulated activity of CEH in CL of cattle and ewes (Biasgaier et al., 1979; Caffrey et al., 1979). Indeed, concentration of CEH is correlated positively with concentration of progesterone in ewes during maximal luteal function (Caffrey et al., 1979). In addition, the concentration of esterified cholesterol in CL is at the nadir during maximal luteal function in cattle (Hafs and Armstrong, 1968). In contrast, concentration of esterified cholesterol is highest in tissues which have reduced steroidogenesis such as bovine luteal tissue collected during late diestrus (Hafs and Armstrong, 1968) and adrenal tissue from aged rats (Popewell and Azhar, 1987). Thus, while esterified cholesterol can serve as a source of cholesterol for steroidogenesis, increased concentration of esterified cholesterol can indicate reduced steroid production. This would explain why the initial concentration of cholesterol esters was correlated negatively with the initial concentration of progesterone in luteal cells

and with production of progesterone during incubation of luteal cells from Restricted animals.

Increased concentration of cholesterol esters in luteal cells of Restricted fed heifers does not appear to be a result of a loss of CEH activity. If luteal cells of Restricted animals lacked CEH activity, the concentration of cholesterol esters in luteal cells would not have declined during incubation of cells. Indeed, it appears that cholesterol esters were hydrolyzed during incubation and increased concentration of free cholesterol in luteal cells of Restricted animals. It is likely that increased concentration of esterified cholesterol in luteal cells of Restricted heifers is due to decreased ability of luteal cells to convert unesterified cholesterol into progesterone. When the delivery of cholesterol to the mitochondrion is reduced or activity of C_{scc} is blocked, the concentration of esterified cholesterol increases in cells (Flint et al., 1973; Schuler et al., 1981; Azhar et al., 1984; Suckling and Stange, 1985). Indeed, compared with positive energy balance, negative energy balance decreased luteal incorporation of ^{3}H-cholesterol in progesterone. Thus, it appears negative energy balance compromised the ability to convert cholesterol into progesterone.

During incubation the concentration of total cholesterol increased in luteal cells from Control but not Restricted animals. However, diet did not affect production of progesterone during incubation. Thus, luteal cells from

Controls appeared to synthesize more cholesterol *de novo* than did cells from Restricted animals. Diet did not affect total HMG-CoA reductase activity (active and inactive forms) in luteal tissue. Thus, ability of luteal cells from Control heifers to produce more cholesterol during a 2 h incubation cannot be explained by change in total activity of HMG-CoA reductase. It is possible that concentration of active form of HMG-CoA reductase (unphosphorylated; Ingebritsen and Gibson, 1980) was higher in luteal cells of Control than in Restricted heifers. Approximately 20 to 40% of HMG-CoA reductase present in luteal tissue of rats (Azhar et al., 1984, 1985, 1988) and rabbits (McLean and Miller, 1988) is the unphosphorylated (active) form. Food deprivation reduced the active state of HMG-CoA reductase in liver of rats (Brown et al., 1979; Zammit and Easom, 1987). Therefore, energy restriction may decrease concentration of active HMG-CoA reductase in luteal tissue. However, level of activation is usually a short term method to alter level of HMG-CoA reductase activity (Beg et al., 1980). Total enzyme present, rather than its level of phosphorylation, is the primary mechanism by which activity of HMG-CoA reductase is altered by long term dietary or hormonal changes in liver (Kleinsek et al., 1978; Brown et al., 1979, Arebalo et al., 1981; Angelin et al., 1984; Popjak et al., 1985) or ovarian tissue (Azhar et al., 1984, 1985, 1988; McLean and Miller, 1988). Duration of dietary restriction in Experiment II lasted several weeks, which is a long term change in diet in regards

to HMG-CoA reductase. Thus, it does not seem probable that reduced concentration of unphosphorylated HMG-CoA reductase in luteal tissue is the cause of reduced *de novo* cholesterol synthesis in Restricted heifers.

In this study, total activity of HMG-CoA reductase averaged 2.90 nMol mevalonic acid produced•min^{-1}•mg^{-1} microsomal protein in luteal tissue. This value is four fold higher than total activity reported in bovine luteal tissue collected from an abattoir (Rodgers et al., 1987a). Methods used to assay HMG-CoA reductase activity appeared similar between studies (Azhar et al., 1984, in Experiment II; Rainey et al., 1986, in Rodgers et al, 1987a). Thus, the exact cause of the discrepancy between studies cannot be determined. However, the half-life of membrane bound HMG-CoA reductase is 1 to 2 h (Luskey, 1988) and concentration of HMG-CoA reductase in bovine CL varies with age of the CL (Rodgers et al., 1987a). Thus, compared with results from Experiment II, total activity of HMG-CoA reductase reported by Rodgers et al. (1987a) may be lower because the enzyme was degraded during transport of tissue to the lab or because of inaccurate estimation of age of CL.

Interestingly, concentration of total HMG-CoA reductase activity in bovine luteal tissue reported in Experiment II is two to ten fold higher than concentration of total enzyme activity reported for luteal tissue of humans (Carr et al., 1981) and pregnant (Azhar et al., 1988) or pseudopregnant (Schuler et al., 1979, 1981) rats. Luteal tissue of rats and

humans depend on lipoproteins in serum for approximately 80% of the cholesterol for steroidogenesis (Gwynne and Strauss, 1982 for review). Lipoproteins also increase production of progesterone by bovine CL *in vivo* and *in vitro* (Pate and Condon, 1982; O'Shaughnessy and Wathes, 1985b; Talvera et al., 1985; Pate et al., 1987; Rodgers et al., 1987a; Williams, 1989). However, greater activity of HMG-CoA reductase in bovine than in human and rat luteal tissue may indicate that steroidogenesis in bovine luteal tissue is more dependent upon *de novo* synthesis of cholesterol to than is luteal tissue of rats or humans. The importance of *de novo* synthesis of cholesterol to steroidogenesis in bovine CL needs more thorough investigation to better understand the basic control of bovine luteal function.

I hypothesized that reduced concentration of acetate and glucose in blood might reduce *de novo* cholesterol synthesis, and production of progesterone during negative energy balance. Concentrations of glucose and acetate added to media were representative of concentrations in blood of cattle during different levels of energy balance ((Lomax and Baird, 1983; de Boer et al., 1985; Harrison and Randel, 1986; McCann and Hansel, 1986). However, there was no consistent relationship between either concentration of acetate or concentration of glucose and luteal production of progesterone *in vitro* to support this hypothesis. Apparently the endogenous concentration of acetate and glucose was adequate to supply the steroidogenic needs of luteal tissue in Control and

Restricted heifers *in vitro*. Also, negative energy balance did not reduce luteal function *in vitro*. Thus, it is not surprising that increased acetate and glucose did not improve luteal function in Restricted animals.

The highest dose of ketones increased production of progesterone in luteal cells from Control animals. Experiment II was not designed to distinquish whether ketones increased substrate for cholesterol biosynthesis or increased energy production from the citric acid cycle (McGarry and Foster, 1980; Bruss, 1989). Utilization of ketones for production of energy or cholesterol has not been previously demonstrated in ovarian tissue. However, muscle, heart and kidney of ruminants use ketones to produce acetyl-CoA for energy (Bell, 1981; Bruss, 1989). Thus, use of ketones for energy production in luteal tissue is plausible. It is also possible that ketones may provide substrate for *de novo* cholesterol synthesis in ruminant ovarian tissue. For *de novo* synthesis of cholesterol, acetyl-CoA generated in the mitochondria from ketone metabolism must condense with oxalacetate to form citrate to be transferred out of the mitochondrion. Citrate is then cleaved by ATP-dependent citrate lyase in the cytosol to free acetyl-CoA. Activity of citrate lyase is low in mammary gland (Bauman and Davis, 1975), liver and adipose tissue of cows (Hanson and Ballard, 1967). However, metabolism of glucose also produces acetyl-CoA in the mitochondrion, yet carbon from glucose (Armstrong and Black, 1966) is incorporated into cholesterol and progesterone in

ovarian tissue of cows. Thus, citrate lyase activity has been indirectly demonstrated in bovine ovarian tissue.

Why did ketones increase production of progesterone in luteal cells of Control but not Restricted heifers? Concentration of ketones in blood of cattle increase several fold during energy restriction (Lomax and Baird, 1983; de Boer et al., 1985). Ketones are rapidly transported through cell membranes (Bruss, 1989). Thus, concentration of ketones in luteal cells of animals in negative energy balance are probably higher than in cells from the Control group. Thus, the positive effects of ketones on *in vitro* luteal function noted in Control heifers may have already been maximized in Restricted heifers.

In conclusion, negative energy balance reduced concentration of progesterone in blood similar to previous reports. This reduction was in part due to reduced weight of CL in Restricted animals. In contrast to previous reports with heifers fed a diet restricted in energy for more than two estrous cycles (Apgar et al., 1975; Imakawa et al., 1983; Villa-Godoy et al., 1990), we found no evidence that negative energy balance for 1.5 estrous cycles reduced basal or LH-induced production of progesterone per luteal cell.

It appears that luteal cells of Restricted animals were able to mobilize their increased stores of esterified cholesterol for production of progesterone *in vitro*. Perhaps the ability of luteal tissue to mobilize cholesterol esters or utilize free cholesterol for production of progesterone is

reduced in animals fed a diet restricted in energy for more than two estrous cycles. Increased initial concentration of esterified cholesterol and decreased incorporation of labeled cholesterol in progesterone in luteal tissue of Restricted heifers are consistent with this suggestion. The ability of restricted energy intake to reduce conversion of cholesterol into progesteone warrants further investigation in heifers that have been underfed for more than two estrous cycles.

GENERAL DISCUSSION

When energy intake is restricted at or below the requirement for maintenance, heifers demonstrate estrous cycles of normal duration for 25 to 30 weeks (Imakawa et al., 1983).. After this time, estrous cycles stop abruptly in most heifers (Imakawa et al., 1983; Imakawa et al., 1986a; Richards et al., 1989). However, preceeding the abrupt transition to the anestrous condition, luteal function is compromised in heifers. For example, the concentration of progesterone in serum was reduced in heifers as early as the first cycle following energy restriction (Hill et al., 1970; Imakawa et al., 1983). The ability of negative energy balance to reduce luteal function in cattle (Hill et al., 1970; Gombe and Hansel, 1973; Imakawa et al., 1983; Villa-Godoy et al., 1988, 1990) is consistent with the metabolic fuel hypothesis of reproductive function: "changes in reproductive status are signaled by changes in the general availability of metabolic fuels" (Schneider and Wade, 1989). The mechanisms by which availability of metabolic fuels affects reproductive function appear to include effects on the hypothalamic-pituitary axis and possibly direct effects on the ovary.

Luteinizing hormone controls development and function of bovine CL. During negative energy balance, reduced

concentration of glucose and insulin in serum may reduce secretion of LH (for review, Butler and Smith, 1989). Indeed, when the duration and severity of energy restriction has caused anovulation, the concentration of LH in serum is reduced (Imakawa et al., 1984; Richards et al., 1989). However, when underfed heifers are still demonstrating estrous cycles, the effect of negative energy balance on the concentration of LH in serum is dependent on the stage of the estrous cycle. For example, during the luteal phase of the estrous cycle, negative energy balance does not reduce the basal concentration of LH in serum or the pulsatile pattern of secretion of LH (Harrison and Randel, 1986; Imakawa et al., 1986b; Richards et al., 1989; Villa-Godoy et al., 1990). Thus, reduced concentration of progesterone in serum induced by negative energy balance, is not due to reduced concentration of LH in serum during the luteal phase. In contrast, during the follicular phase of the estrous cycle, negative energy balance reduces the mean concentration of LH in serum and reduces the amplitude of LH pulses (Imakawa et al., 1986b).

Reduced concentration of LH during the periovulatory period may limit early luteal development (Snook et al., 1969; Farin et al., 1985). Insulin and insulin like growth factor I also stimulate differntiation, mitosis and function of bovine luteal cells (Allen et al., 1981; Mauro et al., 1988; May and Schomberg, 1981; Savion et al, 1981b; O'Shaughnessy and Wathes, 1985a; Poff et al. 1988). Thus, reduced

concentration of insulin and insulin like growth factor I in serum during negative energy balance may also limit luteal development. Restricted luteal development early in the estrous cycle can reduce weight of mature CL. In fact, restricted energy intake consistently reduces weight of bovine CL (Hill et al., 1970; Gombe and Hansel; 1973; Apgar et al., 1975; Spitzer et al., 1978; Harrison and Randel, 1986; Villa-Godoy et al., 1990; Experiment I, II).

Weight of CL is correlated positively with concentration of progesterone in serum in cattle (Spicer et al., 1981; Garverick et al., 1985; Experiment I). Thus, reduced weight of CL may be a mechanism by which negative energy balance reduces the concentration of progesterone in serum. However, I found that negative energy balance increased concentration of receptors for LH on luteal cells. In addition, the concentration of progesterone in serum was not dramatically reduced in Restricted heifers. Thus, when the period of energy restriction was 1.5 estrous cycles, it appears that increased concentration of receptors for LH offsets the effect of reduced weight of CL in Restricted heifers.

Negative energy balance can reduce basal (Villa-Godoy et al., 1990) and LH-stimulated production of progesterone by luteal cells of heifers (Apgar et al., 1975; Imakawa et al., 1983; Villa-Godoy et al., 1990). However, I found restrcited energy intake did not reduce basal or LH-stimulated production of progesterone in either Experiment I or II. Severity of negative energy balance achieved in Experiment II did not

differ from degree of negative energy balance achieved by Villa-Godoy et al. (1990). Therefore, I suggest duration of energy restriction is important to elicit adverse effects of negative energy balance on basal and LH-stimulated production of progesterone.

Perhaps in the transition to anovultation, heifers with inadequate energy intake experience reduced luteal function due to a cascade of events. The first event in this cascade is reduced weight of CL. The next step in the cascade could be reduced ability of luteal cells to convert cholesterol to progesterone. When conversion of cholesterol to progesterone is blocked, the concentration of esterified cholesterol increases in cells. Negative energy balance was associated with increased concentration of esterified cholesterol in luteal tissue. Importantly, concentration of esterified cholesterol was correlated negatively with concentration of progesterone in serum (Experiment I), and Initial and Basal concentration of progesterone in luteal cells (Experiment II). Thus, decreased incorporation of cholesterol into progesterone may be an important step in the reduction of luteal cell function during negative energy balance.

What reduced conversion of free cholesterol to progesterone in CL of Restricted animals? One possibility is that the concentration of the components of the C_{scc} enzyme system in mitochondria are reduced in luteal cells of Restricted heifers. Large luteal cells have a higher concentration of mitochondria than do small luteal cells

(Priedkalns and Weber, 1968a; Kenny et al., 1989). In fact, concentration of C_{scc} is higher in large luteal cells than in small luteal cells (Rodgers et al., 1986a; Gibori et al, 1988). Decreased number of large luteal cells in CL could decrease concentration of C_{scc} in CL. Indeed the ratio of large to small luteal cells was less in CL of heifers in negative energy balance compared with CL of heifers in positive energy balance (Villa-Godoy et al. (1990).

In addition to change in proportion of large luteal cells, problems with development of luteal cells in Restricted animals could affect the concentration of mitochondria in luteal cells. The concentration of mitochondria increased in large ovine luteal cells between day 4 to day 16 of the estrous cycle (Kenny et al., 1989). Since negative energy balance reduced the mature weight of CL, negative energy balance might have also interfered with mitochondrial development in large luteal cells. Thus, negative energy balance may reduce the concentration of C_{scc} in CL.

Since restricted energy intake did not reduce luteal function *in vitro* in either Experiment I or II, the mechanism by which long term negative energy balance reduces basal and LH-stimulated luteal function was not determined. However, the effect of restricted energy intake on receptors for LH and cholesterol metabolism are intriguing. I believe these results provide insight into the fine control of luteal function, and how CL adapt to meet metabolic challenges. Future research questions may include: How does negative

energy balance reduce weight of CL? What is the effect of long term negative energy balance on conversion of cholesterol into progesterone by luteal cells? What is the long term effect of negative energy balance on the concentration of mitochondria and activity of the C_{scc} enzyme system in luteal cells?

SUMMARY AND CONCLUSIONS

Weight of CL was reduced in heifers that were maintaining body weight and in heifers that were losing body weight. Thus, energy restriction impaired development of luteal tissue. However, the concentration of progesterone in serum was only slightly reduced in heifers losing body weight. It appeared that increased concentration of receptors for LH on luteal cells in part offset the negative effect of reduced luteal weight on concentration of progesterone in serum in Restricted heifers.

My original objective was to determine the mechanism by which negative energy balance reduced basal and LH-stimulated production of progesterone from luteal cells *in vitro*. The premise for this objective was that negative energy balance reduced basal and LH-stimulated production of progesterone (Villa-Godoy et al., 1990). I was interested in detecting the initial effects of negative energy balance on luteal function. Restricted energy intake can reduce the concentration of progesterone by the second estrous cycle. Thus, I chose to lutectomize heifers in the second estrous cycle following energy restriction. However, energy balance did not affect basal or LH-stimulated production of progesterone in Experiment I or II. It appears that the difference between

results of Experiment I and II and Villa-Godoy et al. (1990) is due to the shorter duration of energy restriction before lutectomy in Experiment I and II.

The duration of Experiment I and II precluded detection of an effect of dietary energy restriction on luteal production of progesterone. However, in addition to reduced weight of CL the initial effects of dietary energy restriction on luteal function were present. Specifically, dietary energy restriction affected cholesterol metabolism in luteal cells in Experiment I and II. The concentration of esterified cholesterol in luteal cells from Restricted animals was higher than in luteal cells of Control animals. In Experiment I, mobilization of these cholesterol esters was associated with production of progesterone in luteal cells of Restricted animals. I suggest increased duration or severity of negative energy balance would reduce the ability of luteal tissue from Restricted animals to utilize esterified cholesterol esters for steroidogenesis. Data consistent with this suggestion were the observations that initial concentration of cholesterol esters in luteal tissue of Restricted heifers was correlated negatively with concentration of progesterone in serum in Experiment I, and Initial and Basal concentration of progesterone in luteal cells in Experiment II.

Increased concentration of esterified cholesterol in luteal cells of Restricted heifers could be caused by reduced ability of luteal cells to convert cholesterol into progesterone. Indeed, incorporation of cholesterol into

progesterone by luteal cells was reduced in Restricted fed animals in Experiment II.

De novo synthesis of cholesterol in luteal cells of Control animals was higher than in luteal cells of Restricted animals. The reason for this is not clear. Diet did not affect the concentration of HMG-CoA reductase activity in luteal tissue of heifers. Thus, HMG-CoA reductase can not be used to explain the difference in *de novo* synthesis of cholesterol between Control and Restricted heifers.

My initial hypothesis was that negative energy balance would the concentration of cholesterol in tissue and thereby limit ability of luteal cells to produce progesterone. My data do not support for this hypothesis since initial concentration of cholesterol in luteal tissue did not differ between diets. Thus, I rejected this hypothesis. Instead, based on cholesterol metabolism in luteal cells, I hypothesize that long term negative energy balance affects luteal function by reducing the activity of the C_{scc} enzyme system in luteal cells.

Large luteal cells have a higher concentration of C_{scc} than do small luteal cells (Rodgers et al., 1986a). Long term negative energy balance reduced CL weight and number of large luteal cells present (Villa-Godoy et al., 1990). Thus, negative energy balance may have reduced luteal content and concentration of C_{scc}. In addition, the concentration of mitochondria in large luteal cells increases almost two fold as the CL matures (Kenny et al., 1989). Factors which affect

development of large luteal cells, and also size of CL may also affect development of mitochondria in large luteal cells. Thus, negative energy balance may reduce the concentration of C_{scc} in luteal cells by reducing the number and development of large luteal cells.

In conclusion, the most dramatic effect of restricted energy intake was reduced weight of CL. In addition, restricted energy intake changed some aspects of cholesterol metabolism. During long term energy restriction the changes in cholesterol metabolism may be exacerbated and subsequently limit production of progesterone by luteal tissue.

APPENDICES

APPENDIX 1: VALIDATION OF LH BINDING ASSAY

Assays to determine number and equilibrium association constants (K_a) of binding sites for hCG have been validated in our laboratory for homogenized luteal tissue (Spicer et al., 1981) but not with dispersed luteal cells . Specificity of binding sites in luteal cells to ^{125}I-hCG was tested by competition with unlabeled hCG. To determine if number of cells incubated affected binding of ^{125}I-hCG, .2 x 10^6 to 6 x 10^6 cells were incubated with ^{125}I-hCG (20,000 cpm). Scatchard analyses (Smith and Sestili, 1980) with 5 mg luteal homogenates, .25 x 10^6 and 1 x 10^6 luteal cells were used to examine effects of type of tissue and number of luteal cells on estimates of K_a and numbers of unoccupied binding sites.

To validate radioreceptor assays for dispersed luteal cells, a pool of dissociated cells was prepared from CL of nonpregnant heifers. In addition, a pool of homogenized luteal tissue was prepared as described previously (Spicer et al., 1981) from CL collected from nonpregnant cattle at slaughter. All cells and homogenates used to validate the radioreceptor assay were stored frozen in 20% glycerol-PBS at -70°C until day of use.

Specificity of binding sites on luteal cells to hCG was demonstrated because addition of unlabelled hCG inhibited

binding of ^{125}I-hCG (5 x 10^4 cpm_{crt}) to luteal cells (Figure 10). In addition, specific binding of ^{125}I-hCG was saturable (Figure 11). The percent of specific binding of ^{125}I-hCG increased linearly (Gill, 1978a) as number of incubated cells increased from .2 x 10^6 to 1 x 10^6 cells (Figure 12). Similarly, incubation of .25 x 10^6 or 1 x 10^6 cells did not affect estimation of concentration of numbers of binding sites for LH determined from four point saturation curves (33.2 versus 32.2 fMol/mg protein, respectively).

Luteal cells (0.5 x 10^6), or luteal homogenates (5 mg) were incubated with increasing concentrations of ^{125}I-hCG (.2 x $10^4 cpm_{crt}$ to 25 x $10^4 cpm_{crt}$) in the presence (background binding) or absence (total binding) of 40 µg LH and Scatchard analyses were performed (Figure 13). Type of tissue preparation did not affect estimation of K_a (2.3 versus 2.6 x $10^{10} M^{-1}$ cells and homogenates, respectively). Estimated concentration of binding sites were not different between the two tissue preparations (31.3 versus 32.1 fMol/µg protein, homogenates and luteal cells respectively). In addition, concentration of binding sites and affinity of binding sites determined with luteal cells are similar to values determined with homogenized bovine CL collected during mid-diestrus (Rao et al., 1979).

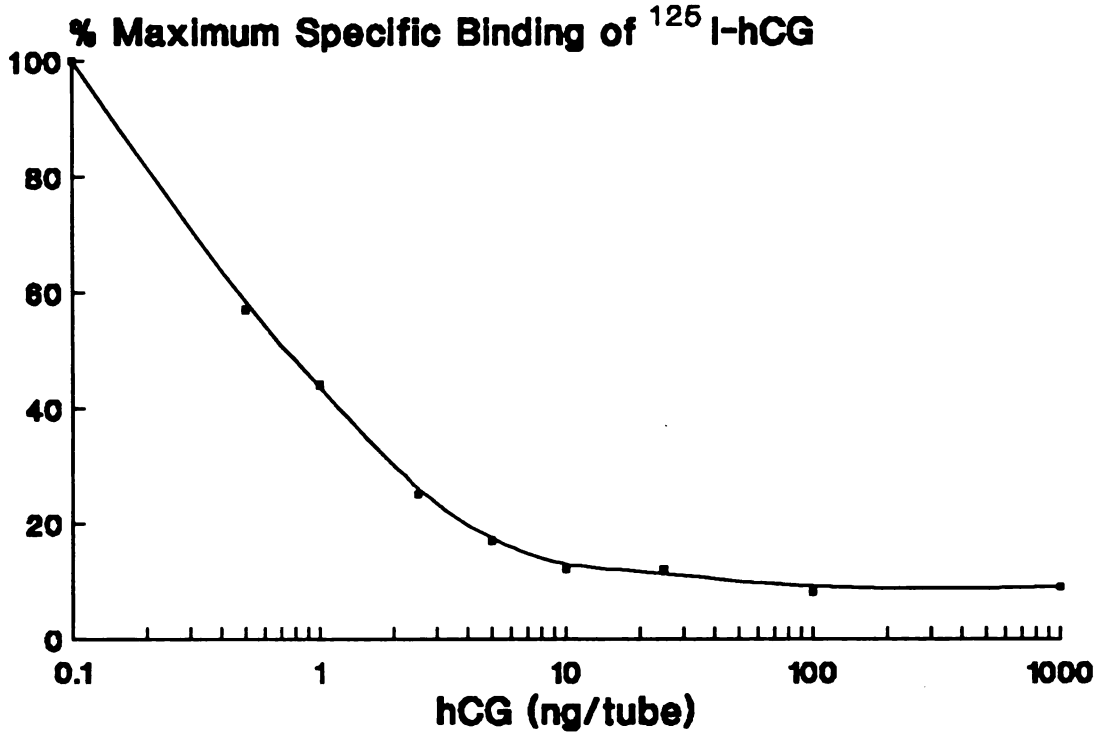


Figure 10. Displacement of ^{125}I-hCG (5 x 10^4cpm$_{act}$) by increasing dose of hCG. Specific binding is listed as a percent of maximum. Determinations were done in triplicate with .25 x 10^6 cells/tube at each point.

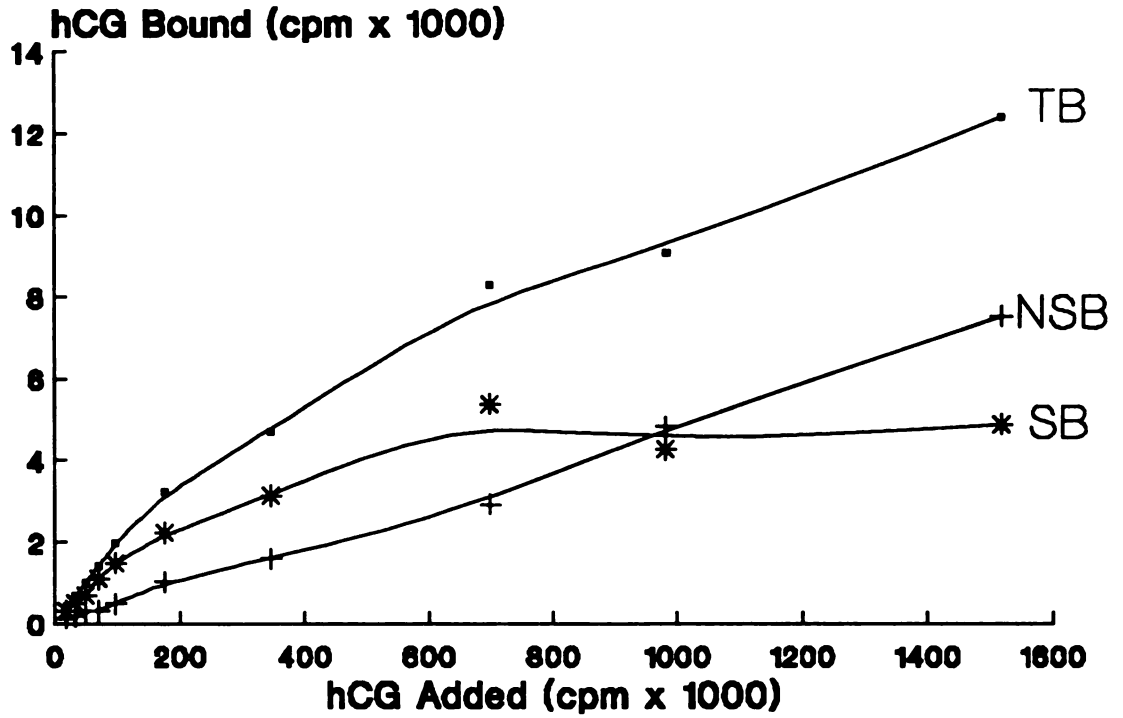


Figure 11. Specific binding of ^{125}I-hCG to 1 x 10^6 luteal cells. Specific binding (SB, *) = total binding (TB, ■) minus nonspecific binding (NSB, +).

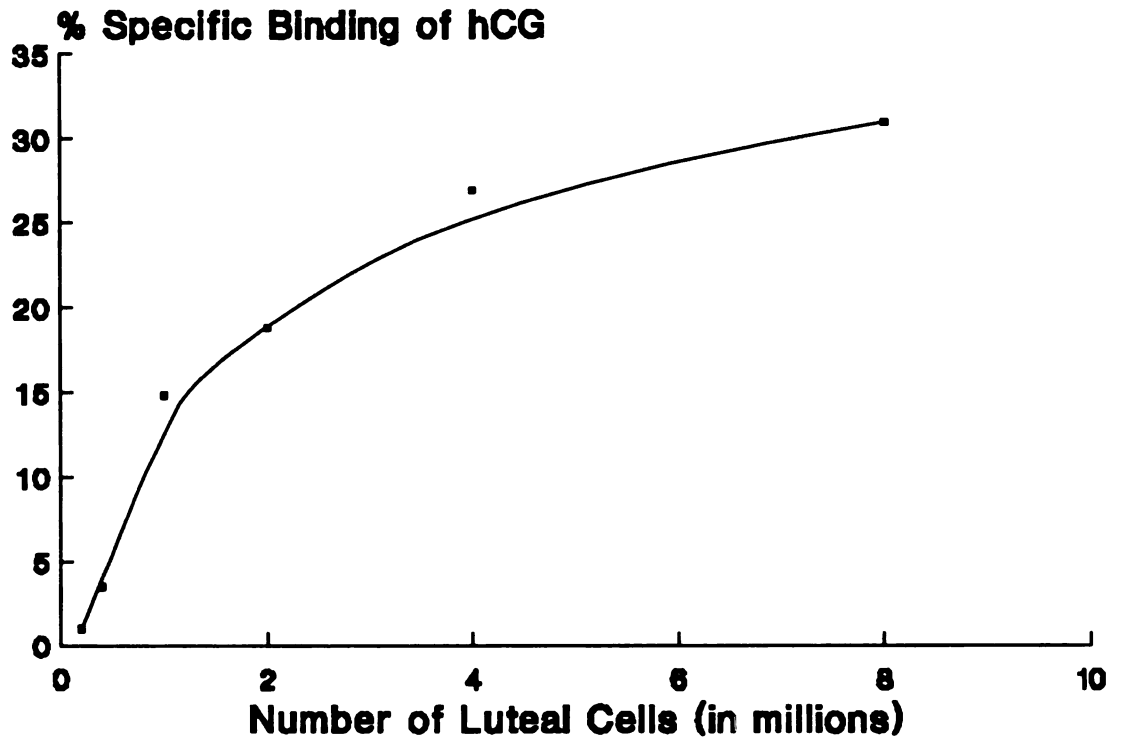
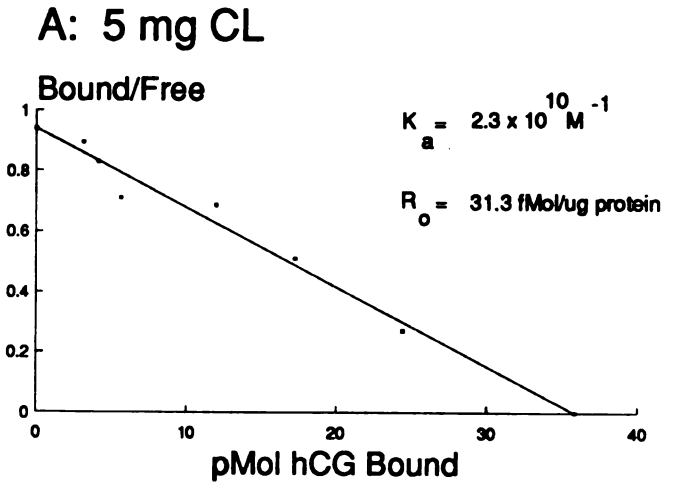
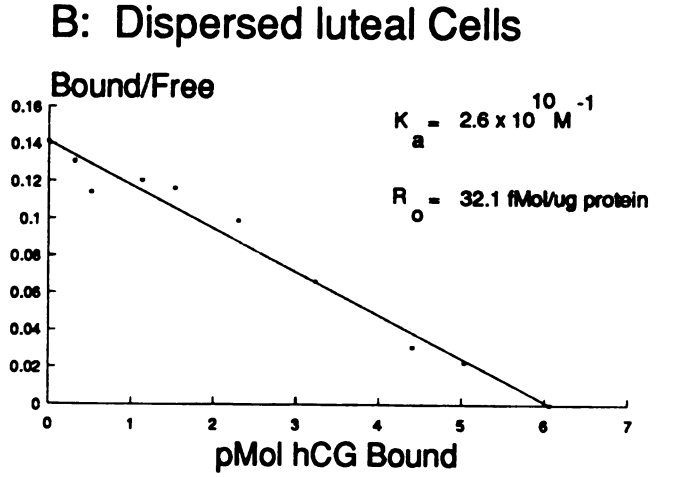


Figure 12. Effect of the number of luteal cells on percent specific binding of ^{125}I-hCG. Amount of ^{125}I-hCG (25,000 cpm) per tube was constant.

Figure 13. Estimation by Scatchard analysis of the number of unoccupied binding sites for LH in luteal homogenates (5 mg; A), and dispersed luteal cells (.5 x 10^6 cells; B). R_o represents the X axis intercept. K_a represnts the inverse of the slope.

Figure 13.


A: 5 mg CL
Bound/Free
K_a = 2.3 x 10^{10} M^{-1}
R_o = 31.3 fMol/ug protein
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
pMol hCG Bound


B: Dispersed luteal Cells
Bound/Free
K_a = 2.6 x 10^{10} M^{-1}
R_o = 32.1 fMol/ug protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
2
3
4
5
6
7
pMol hCG Bound

APPENDIX 2: VALIDATION OF SEPARATION OF STEROIDS BY HPLC

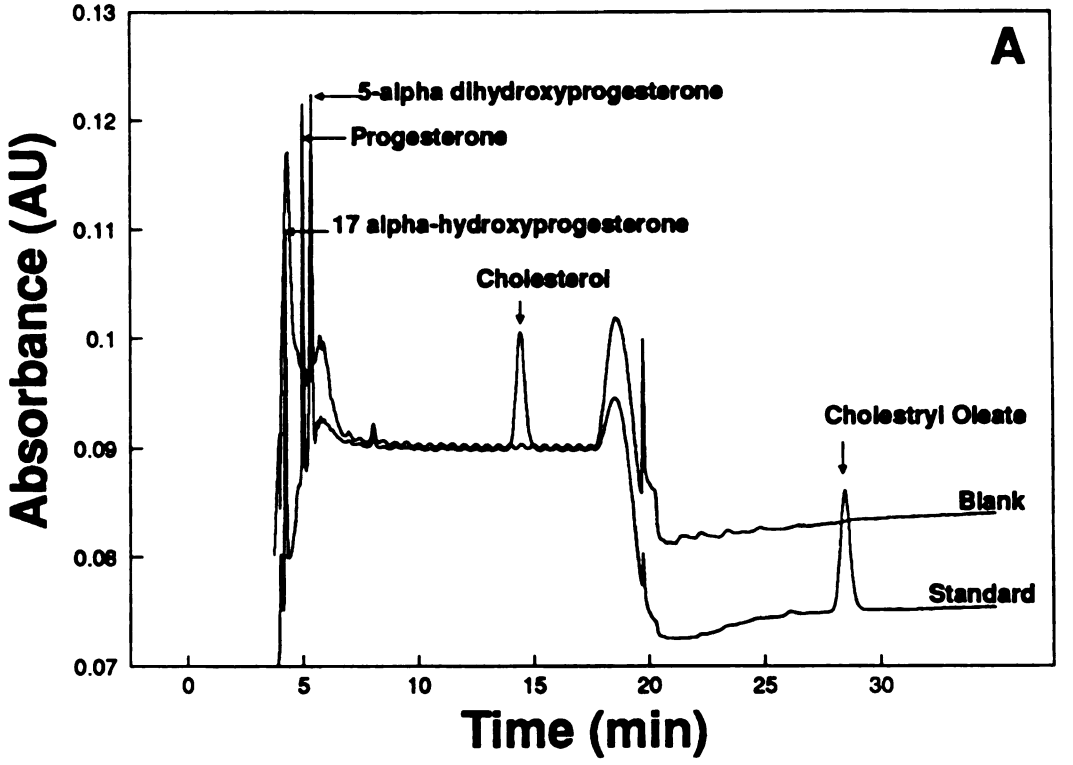
Standard solutions of 17-α hydroxyprogesterone, progesterone, 5 α-dihydroxyprogesteroneprogestins, cholesterol and cholesteryl oleate were prepared separately and in combination in acetonitrile. Standard solutions were used to determine retention times of the compounds and degree of separation of compounds. Solutions of standards with increasing concentrations were used to determine the sensitivity of the method and to allow quantification. Linear regression lines for the concentration of standard and absorbance of standards were constructed for each standard using Maxima® software (Waters, Division of Millipore, Milford, MA). Results are presented in Table 12. Absorbance of each standard was recorded at wavelengths determined to give maximum absorbance (Waters Programmable 994 photodiode array detector) of purified cholesterol, cholesteryl oleate (242 nanometers, nm) or progesterone (194 nm) by spectral analysis. Regression lines constructed for these compounds were linear ($r^2 \approx .999$). Sensitivity to detect compounds of interest were: 25 ng progesterone (A); 50 ng cholesterol (B); 25 ng cholesterol oleate (C). Retention times determined with each standard injected individually were: 17-α hydroxyprogesterone, 4.21 min; progesterone, 4.96

min; 5α-dihydroxyprogesterone, 5.28 min; cholesterol, 14.69 min; cholesterol oleate, 29.43 min. Absorbance profiles at 194 nm (A) and 242 (B) and retention times of a mix containing 500 ng each of 17-α hydroxyprogesterone, progesterone, 5 α-dihydroxyprogesterone, cholesterol and cholesteryl oleate are shown in Figure 14.

Counts per minute in fractions after injection of ^{3}H-progesterone or ^{3}H-cholesterol and retention times of standard preparations were compared. While over 95% of ^{3}H-progesterone coeluted with progesterone standard, only 88% of ^{3}H-cholesterol coeluted with cholesterol standard (mean of four determinations; Figure 15). Counts per minute which did not coelute with cholesterol standard were assumed to be impurities present in the original preparation of ^{3}H-cholesterol. Counts per minute calculated to be a result of impurities in ^{3}H-cholesterol were subtracted from cpm in fractions from samples to remove background prior to statistical analyses of data as described in Materials and Methods and Statistical Analyses for Experiment II.

Figure 14. Absorbance profiles at 194 (A) and 242 (B) nm and retention times of a variety of steroids. The absorbance profile of a mixture containing 500 ng each of 17-α hydroxyprogesterone, progesterone, 5α-dihydroxyprogesterone, cholesterol and cholesteryl oleate in 20 µl acetonitrile is shown in A and B. Absorbance following an injection of 20 µl acetonitrile is included as a blank.

Figure 14.


A
5-alpha dihydroxyprogesterone
Progesterone
17 alpha-hydroxyprogesterone
Cholesterol
Cholestryl Oleate
Blank
Standard
Absorbance (AU)
Time (min)
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0
5
10
15
20
25
30

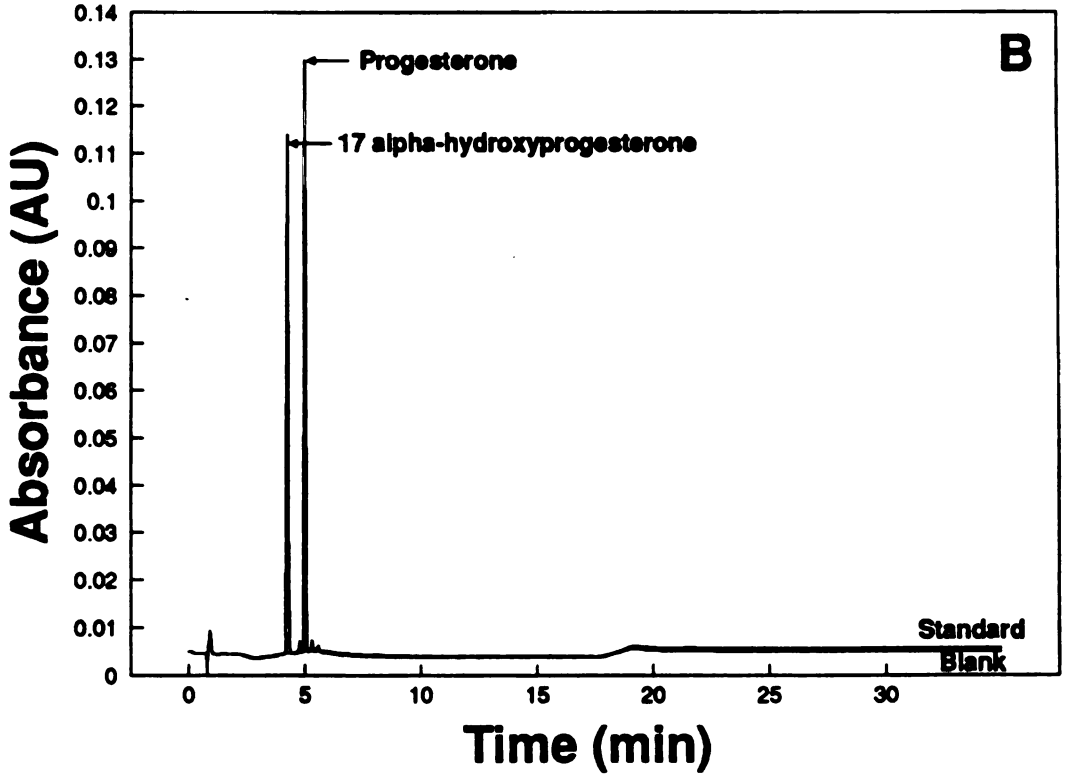

B
Progesterone
17 alpha-hydroxyprogesterone
Standard
Blank
Absorbance (AU)
Time (min)
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
25
30

Table 12. Characteristics of HPLC Analysis of Standard Solutions.

Compound	Monitored Wavelength (nm)	Retention Time (min)	Standard Curve Line Equation[a]	r^{2b}
17-α Hydroxyprogesterone	242	4.21	$0.099 + (1.55 \times 10^{-3}) \times R$	.999
Progesterone	242	4.96	$-1.479 + (1.15 \times 10^{-3}) \times R$	.999
5α-Dihydroxyprogesterone	194	5.28	$-38.544 + (5.24 \times 10^{-3}) \times R$	.999
Cholesterol	194	14.69	$2.828 + (2.36 \times 10^{-3}) \times R$	.998
Cholesteryl Oleate	194	29.43	$-3.864 + (6.45 \times 10^{-2}) \times R$	.999

[a] Dilutions (25 to 500 ng/20 µl acetonitrile) of standards were used to construct standard curves. Line equations for concentration of compound (ng/injection) versus absorbance (AU) were calculated by Maxima® software (Waters, Milford, MA). R stands for area under the response curve of peak response.

[b]Coefficient of determination for constructed lines.

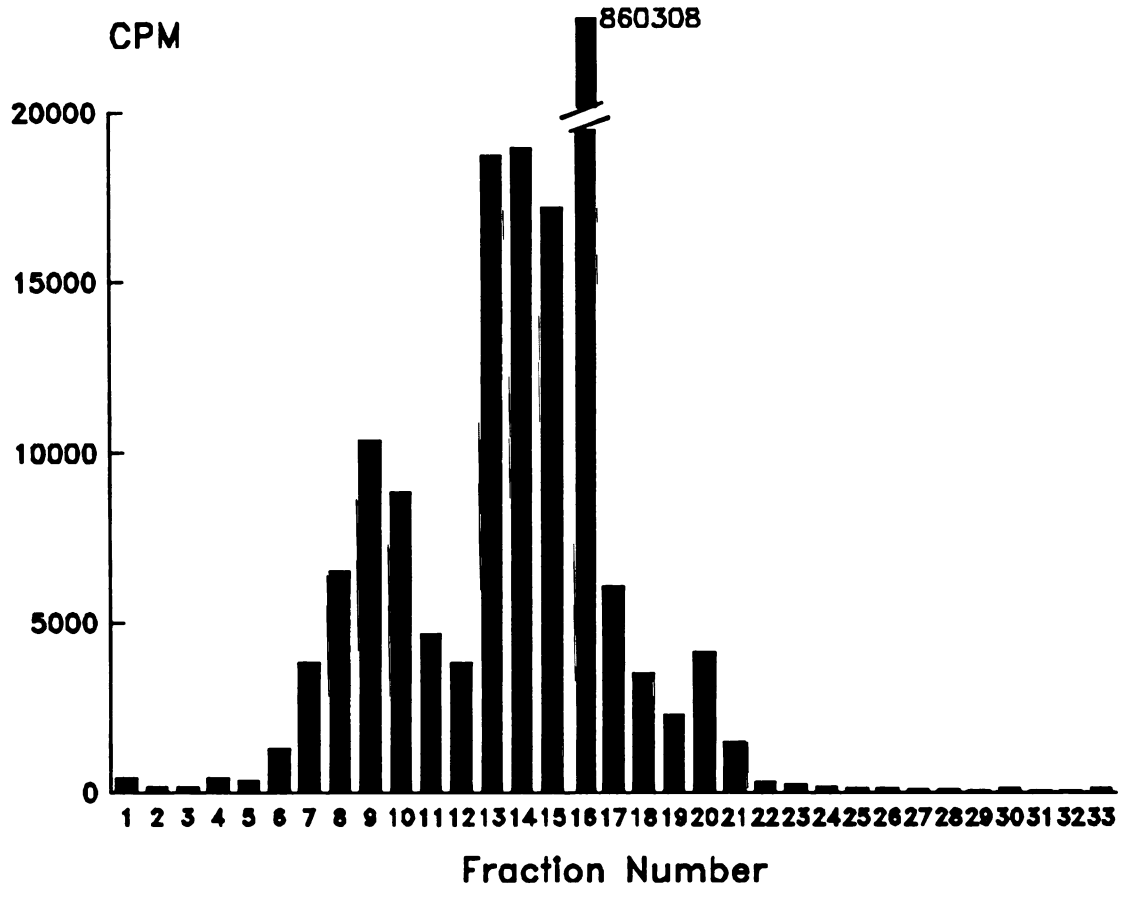


Figure 15. Counts per minute in fractions eluting following injection of 1.4 µCi [1,2,6,7-^{3}H]cholesterol. Bars represent the mean of 4 determinations.

LITERATURE CITED

LITERATURE CITED

Alila HW, Hansel W. Origin of different cell types in the bovine corpus luteum as characterized by specific monoclonal antibodies. Biol Reprod 1984; 31:1015-1025.

Allen WR, Nilsen-Hamilton M, Hamilton RT. Insulin and growth factors stimulate rapid posttranslational changes in glucose transport in ovarian granulosa cells. J Cell Physiol 1981; 105:15-24.

Anderson JM, Dietschy JM. Absolute rates of cholesterol synthesis in extrahepatic tissues measured with ^{3}H-labeled water and ^{14}C-labeled substrates. J Lipid Res 1979: 20:740-752.

Anderson JM, Dietschy JM. Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. J Biol Chem 1978; 253;9024-9032.

Angelin B, Einarsson K, Liljeqvist L, Nilsell K, Heller RA. 3-Hydroxy-3-methylglutaryl coenzyme A reductase in human liver microsomes: active and inactive forms and cross-reactivity with antibody against rat liver enzyme. J Lipid Res 1984; 25:1159-1166.

Apgar J, Aspros D, Hixon JE, Saatman RR, Hansel W. Effect of restricted feed intake on the sensitivity of the bovine corpus luteum to LH in vitro. J Anim Sci 1975; 41:1120-1123.

Arebalo RE, Hardgrave JE, Scallen TJ. The *in vivo* regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem 1981; 256:571-574.

Armstrong DT, Black DL. Control of progesterone biosynthesis in the bovine corpus luteum: effects of luteinizing hormone and reduced nicotinamide-adenine dinucleotide phosphate. Can J Biochemistry 1968; 46:1137-1145.

Armstrong DT, Black DL. Influence of luteinizing hormone on corpus luteum metabolism and progesterone biosynthesis throughout the bovine estrous cycle. Endocrinology 1966; 78:937-944.

Armstrong DT, Lee TP, Miller LS. Stimulation of progesterone biosynthesis in bovine corpora lutea by luteinizing hormone in the presence of an inhibitor of cholesterol synthesis. Biol Reprod 1970; 2:29-36.

Ascoli M, Freeman DA. Sources of cholesterol used for steroid biosynthesis in cultured leydig tumor cells. In: Strauss JF, Menon KMJ (ed.), Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues. Philadelphia, PA: George F Stickley Co; 1985:21-32.

Aviram M, Bierman E, Chait A. Modification of low density lipoprotein by hepatic lipase induces enhanced uptake and cholesterol accumulation in cells. J Biol Chem 1988; 263:15416-15422.

Azhar S., Chen YDI, Reaven GM. Gonadotropin modulation of 3-hydroxy-3-methlyglutaryl coenzyme A reductase activity in desensitized luteinized rat ovary. Biochemistry 1984; 23 :4533-4538.

Azhar S, Khan I, Chen YDI, Reaven GM, Gibori G. Regulation of luteal cell 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by estradiol. Biol Reprod 1985; 32:333-341.

Azhar S. Khan I, Puryear T, Chen YDI, Gibori G. Luteal cell hydroxy-3-methylglutraryl conenzyme-A reductase activity and cholesterol metabolism throughout pregnancy in the rat. Endocrinology 1988; 123:1495-1503.

Azhar S, Menon KMJ. Cyclic adenosine, 3',5'-monophosphate and luteinizing hormone stimulated protein kinase from bovine corpus luteum: Evidence for activation through separate mechanisms. FEBS Letters 1975; 51:25-28.

Azhar S, Menon KMJ. Receptor-mediated gonadotropin action in the ovary. J Biol Chem 1981; 256:6548-6555.

Balasubramaniam S, Goldstein JL, Faust JR, Brunschede GY, Brown MS. Lipoprotein-mediated regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesterol ester metabolism in the adrenal gland of the rat. J Biol Chem 1977; 252:1771-1779.

Balboni GC. Structural changes: ovulation and luteal phase. In: Serra GD (ed.), The Ovary. New York: Raven Press; 1983:123-141.

Bartley JC. Lipid metabolism and its diseases. In: Kaneko JJ (ed.), Clinical Biochemistry of Domestic Animals, 4th ed., San Diego, CA: Academic Press, Inc; 1989:106-141.

Bartol FF, Thatcher WW, Bazer FW, Kimball FA, Chenault JR, Wilcox CJ, Roberts RM. Effects of the estrous cycle and early pregnancy on bovine uterine, luteal, and follicular responses. Biol Reprod 1981; 25:759-776.

Bauman DE, Currie WB. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci 1980; 63:1514-1529.

Bauman DE, Davis CL. Regulation of lipid metabolism. In: McDonald IW, Warner ACJ (ed.), Digestion and Metabolism in the Ruminant. Proceedings of the IV International Symposium on Ruminant Physiology; Sydney, Australia; 1975:496-509.

Beg ZH, Stonik JA, Brewer HB Jr. *In vitro* and *in vivo* phosphorylation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase and its modulation by glucagon. J Biol Chem 1980; 255:8541-8545.

Bell AW. Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. In: Christie WW (ed.), Lipid Metabolism in Ruminant Animals. New York, NY: Pergamon Press; 1981:363-410.

Bisgaier CL, Treadwell CR, Vahouny GV. Activation of sterol ester hydrolase of bovine corpus luteum by $N^6,O^{2'}$-Dibutyryl cyclic adenosine 3′:5′-phosphate. Lipids 1979; 14:1-4.

Bradley WA, Gianturco SH. ApoE is necessary and sufficient for the binding of large triglyceride-rich lipoproteins to the LDL receptor; apoB is unnecessary. J Lipid Res 1986; 27:40-48.

Brier BH, Gluckman PD, Bass JJ. Influence of nutritional status and oestradiol-17β on plasma growth hormone, insulin-like growth factors-I and -II and the response to exogenous growth hormone in young steers. J Endocr 1988; 118:243-250.

Brody RI, Black VH. Acyl-coenzyme A:cholesterol acyltransferase and cholesterol ester hydrolase in the outer and inner cortices of the guinea pig adrenal: effects of adrenocorticotropin and dexamethasone. Endocrinology 1988; 122:1722-1731.

Brown BW, Cognie Y, Chemineau P, Poulin N, Salama OA. Ovarian capillary blood flow in seasonally anoestrous ewes induced to ovulate by treatment with GnRH. J Reprod Fert 1988; 84:653-658.

Brown MS, Dana SE, Goldstein JL. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts. J Biol Chem 1974; 249:789-796.

Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986; 232:34-47.

Brown MS, Goldstein JL, Dietschy JM. Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat. J Biol Chem 1979; 254:5144-5149.

Brumby PE, Anderson M, Tuckley B, Storry JE. Lipid metabolism in the cow during starvation-induced ketosis. Biochem J 1975; 146:609-615.

Bruss ML. Ketogenesis and ketosis. In: Kaneko JJ(ed.), Clinical Biochemistry of Domestic Animals, 4th ed. San Diego, CA: Academic Press, Inc; 1989:86-105.

Budnik LT, Mukhopodhyay AK. Desensitization of LH-stimulated cyclic AMP accumulation in isolated bovine luteal cells - effect of phorbol ester. Mol Cell Endocrinol 1987; 54:51-61.

Butler WR, Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci 1989; 72:767-783.

Caffrey JL, Fletcher PW, Diekman MA, O'Callaghan PL, Niswender GD. The activity of ovine luteal cholesterol esterase during several experimental conditions. Biol Reprod 1979; 21:601-608.

Carew TE, Pittman RC, Seinberg D. Tissue sites of degradation of native and reductively methylated [^{14}C]sucrose-labeled low density lipoprotein in rats. Contributions of receptor dependent and receptor independent pathways. J Biol Chem 1982; 257:8001-8008.

Caron MG, Goldstein S, Savard K, Marsh JM. Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in the bovine corpus luteum. J Biol Chem 1975; 250:5137-5143.

Carr BR, Sadler RK, Rochelle DB, Talmach MA, MacDonald PC, Simpson ER. Plasma lipoprotein regulation of progesterone biosynthesis by human corpus luteum tissue in organ culture. J Clin Endocrinol Metab 1981; 52: 875-881.

Carstairs JA, Morrow DA, Emery RS. Postpartum reproductive function of dairy cows as influenced by energy and

phosphorus status. J Anim Sci 1980; 51:1122-1130.

Chait A. The role of lipoprotein receptors in lipid transport and in the pathogenesis of the hyperlipoptroteinemias. In: Cohen MP, Foa PP (ed.), Special Topics in Endocrinology and Metabolism, vol 5. New York, NY: Alan R Liss, Inc.; 1983:1-53.

Chaiyabutr N, Faulkner A, Peaker M. The utilization of glucose for the synthesis of milk components in the fed and starved lactating goat *in vivo*. Biochem J 1980; 186:301-308.

Chang T-Y, Limanek JS. Regulation of cytosolic acetoacetyl coenzyme A thiolase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and mevalonate kinase by low density lipoprotein and by 25-hydroxy cholesterol in chinese hamster ovary cells. J Biol Chem 1980; 255:7787-7795.

Channing CP. Progesterone and estrogen secretion by cultures monkey ovarian cell types: influences of follicular size, serum luteinizing hormone levels, and follicular fluid estrogen levels. Endocrinology 1980; 107:342-352.

Chapman MJ. Animal lipoproteins: chemistry, structure, and comparative aspects. J Lipid Res 1980; 21:789-853.

Chew BP, Erb RE, Fessler JF, Callahan CJ, Malven PV. Effects of ovariectomy during pregnancy and of prematurely induced parturition on progesterone, estrogens, and calving traits. J Dairy Sci 1979; 62:557-566.

Cohen P. The structure and regulation of protein phosphatases. In: Richardson CC (ed.), Annu Rev Biochem, vol 58. Palo Alto, CA: Annual Reviews; 1989:453-508.

Condon WA, Pate JL. Influence of serum and its lipoprotein fractions on progesterone synthesis and secretion by bovine luteal tissue *in vitro*. Biol Reprod 1981; 25:950-957.

Corah LR, Quealy AP, Dunn TG, Kaltenbach CC. Prepartum and postpartum levels of progesterone and estradiol in beef heifers fed two levels of energy. J Anim Sci 1974; 39:380-385.

Cordle SR, Clegg RA, Yeaman SJ. Purification and characterization of bovine lipoproteins: resolution of high density and low density lipoproteins using heparin-Sepharose chromatography. J Lipid Res 1985; 26:721-725.

Darbon JM, Ursely J, Leymarie P. Stimulation by LH of cyclic AMP-dependent protein kinase activity in bovine corpus luteum slices. FEBS Letters 1976. 63;159-163.

Darbon JM, Ursely J, Mangue N, Leymarie P. Stimulation by LH of cytosolic protein phosphorylation in bovine luteal cells. FEBS Letters 1980; 113:120-124.

de Boer G, Trenkle A, Young JW. Glucagon, insulin, growth hormone, and some blood metabolites during energy restriction ketonemia of lactating cows. J Dairy Sci 1985; 68:326-337.

Diekman MA, O'Callaghan PO, Nett TM, Niswender GD. Validation of methods and quantification of luteal receptors for LH throughout the estrous cycle and early pregnancy in ewes. Biol Reprod 1978; 19:999-1009.

Dielman SJ, Blankenstein DM. Progesterone-synthesizing ability of preovulatory follicles of cows relative to the peak of LH. J Reprod Fert 1985; 75:609-615.

Dietschy JM, Brown MS. Effect of alterations of the specific activity of the intracellular acetyl CoA pool on apparent rates of hepatic cholesterogenesis. J Lipid Res 1974; 15:508-516.

Donaldson L, Hansel W. Histological study of bovine corpora lutea. J Dairy Sci 1965; 48:905-909.

Dubois RN, Simpson ER, Kraemer RE, Waterman MR. Induction of synthesis of cholesterol side chain cleavage cytochrome P-450 by adrenocorticotropin in cultured bovine adrenocortical cells. J Biol Chem 1981a; 256:7000-7005,

Dubois RN, Simpson ER, Tuckey J, Lambeth JD, Waterman MR. Evidence for a higher molecular weight precursor of cholesterol side-chain-cleavage cytochrome P-450 and induction of mitochondrial and cytosolic proteins by corticotropin in adult bovine adrenal cells. Proc Natl Acad Sci 1981b; 78:1028-1032.

Dufour J, Whitmore HL, Ginther OJ, Casida LE. Identification of the ovulating follicle by its size on different days of the estrous cycle in heifers. J Anim Sci 1972; 34:85-87.

Edwards PA, Lemongello D, Kane J, Shechter I, Fogleman AM. Properties of purified rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and regulation of enzyme activity. J Biol Chem 1980; 255:3715-3725.

Eisenberg S. High density lipoprotein metabolism. J Lipid

Res 1984; 25:1017-1058.

Estergreen VL Jr, Frost OL, Gomes WR, Erb RE, Bullard Jf. Effect of ovariectomy on pregnancy maintenance and parturition in dairy cows. J Dairy Sci 1967; 50:1293-1295.

Fairchild DL, Pate JL. LH maintains steroidogenic cell number in cultured bovine luteal cells. J Anim Sci 1987; 65(suppl 1): 364 (abstract 407).

Farin CE, Moeller CL, Sawyer HR, Gamboni F, Niswender GD. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol Reprod 1986; 35:1299-1308.

Farin CE, Schwall RH, Gamboni F, Sawyer HR, Niswender GD. Effect of LH and hCG on size distribution of luteal cells in the cycling ewe. Biol Reprod 1985; 32(suppl 1): 44 (abstract 13).

Farkash Y, Timberg R, Orly J. Preparation of antiserum to rat cytochrome P-450 cholesterol side chain cleavage, and its use for ultrastructural localization of the immunoreactive enzyme by protein A-gold technique. Endocrinology 1986; 118:1353-1365.

Faust MA, McDaniel BT, Robison OW, Britt JH. Environmental and yield effects on reproduction in primiparous Holsteins. J Dairy Sci 1988; 71:3092-3099.

Feingold KR, Wiley MH, Moser AH, Lear SR, Siperstein MD. Activation of HMG-CoA reductase by microsomal phosphatase. J Lipid Res 1983; 23:290-296.

Field FJ, Erickson SK, Shrewsbury MA, Cooper AD. 3-Hydroxy-3-methylglutaryl coenzyme A reductase from rat intestine: subcellular localization and *in vitro* regulation. J Lipid Res 1982; 23:105-113.

Fitz TA, Mayan MH, Sawyer HR, Niswender GD. Characterization of two steroidogenic cell types in the ovine corpus luteum. Biol Reprod 1982; 27:703-711.

Flint APF, Denton RM. Glucose metabolism in the superovulated rat ovary *in vitro*. Biochem J 1969; 112:243-254.

Flint APF, Denton RM. Metabolism of endogenous sterol ester by the superovulated rat ovary *in vitro*. Biochem J 1970: 116:79-82.

Flint APF, Grinwich DL, Armstrong DT. Control of ovarian cholesterol ester biosynthesis. Biochem J 1973;

132:313-321.

Folman Y, Rosenburg M, Herz Z, Davidson M. The relationship between plasma progesterone concentration and conception and conception in *post-partum* dairy cows maintained on two levels of nutrition. J Reprod Fert 1973; 34:267-278.

Freeman DA. Cyclic AMP mediated modification of cholesterol traffic in leydig tumor cells. J Biol Chem 1987; 262:13061-13068.

Freeman DA. Plasma membrane cholesterol: removal and insertion into the membrane and utilization as substrate for steroidogenesis. Endocrinology 1989; 124:2527-2534.

Funkenstein B, Waterman MR, Masters BSS, Simpson ER. Evidence for the presence of cholesterol side chain cleavage cytochrome P-450 and adrenodoxin in fresh granulosa cells. J Biol Chem 1983; 258:10187-10191.

Funkenstein B, Waterman MR, Simpson ER. Induction of synthesis of cholesterol side chain cleavage cytochrome P-450 and adrenodoxin by follicle-stimulating hormone, 8-bromo-cyclic AMP, and low density lipoprotein in cultures bovine granulosa cells. J Biol Chem 1984; 259:8572-8577.

Garret JE, Geisert RD, Morgan GL, Wettemann RP, Zavy MT, Gries LK, Buchanan DS. Effect of exogenous progesterone on cycle length, embryonic development and maintenance of pregnancy in the bovine. J Anim Sci 1987; 65(suppl 1):418 (abstract 534).

Garverick HA, Smith MF, Elmore RG, Morehouse GL, Agudo LS, Zahler WL. Changes and interrelationships among luteal LH receptors, adenylate cyclase activity and phosphodiesterase activity during the bovine estrous cycle. J Anim Sci 1985; 61:216-223.

Gebhard RL, Stone BG, Prigge WF. 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity in the human gastrointestinal tract. J Lipid Res 1985; 26:47-53.

Ghosh DK, Dunham WR, Sands RH, Menon KMJ. Regulation of cholesterol side-chain cleavage enzyme activity by gonadotropin in rat corpus luteum. Endocrinology 1987; 121:21-27.

Ghosh DK, Menon KMJ. Induction of high-density-lipoprotein receptors in rate corpus luteum by human choriogonadotropin. Biochem J 1987; 244:471-479.

Gibori G, Khan I, Warshaw ML, McLean MP, Puryear TK, Nelson S, Durkee TJ, Azhar S, Steinschneider A, Rao MC. Placental-derived regulators and the complex control of luteal cell function. In: Clark JH (ed.), Recent Progress in Hormone Research, vol 44. San Diego, CA: Academic Press; 1988:377-429.

Gill JL. Design and analysis of experiments, vol 1 Ames, Iowa: The Iowa State University Press; 1978a: 156-158.

Gill JL. Design and analysis of experiments, vol 2 Ames, Iowa: The Iowa State University Press; 1978b:169-260.

Gill JL. Repeated measurement: sensitive tests for experiments with few animals. J Anim Sci 1986; 63:943-954.

Gill JL. Standard errors for split-split-plot experiments with repeated measurements of animals. J Anim Breed Genet 1988; 105:329-336.

Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fert 1989; 87:223-230.

Goldring NB, Durica JM, Lifka J, Hedin L, Ratoosh SL, Miller WL, Orly J, Richards JS. Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. Endocrinology 1987; 120:1942-1950.

Goldstein JL, Brown MS. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J Lipid Res 1984; 25:1450-1461.

Golos TG, August AM, Strauss JF. Expression of low density lipoprotein receptor in cultured human granulosa cells: regulation by human chorionic gonadotropin, cyclic AMP, and sterol. J Lipid Res 1986; 27:1089-1096.

Gombe S, Hansel W. Plasma luteinizing hormone (LH) and progesterone levels in heifers on restricted energy intakes. J Anim Sci 1973; 37:728-733.

Goshima K, Masuda A, Owaribe K. Insulin-induced formation of ruffling membranes of KB cells and its correlation with enhancement of amino acid transport. J Cell Biol 1984; 98:801-809.

Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F, Bohlen P.

Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 1985; 117:2283-2381.

Gospodarowicz D, Gospodarowicz F. Bovine luteal cells in tissue culture. Exptl Cell Res 1972; 75;353-362.

Gospodarowicz D, Gospodarowicz F. The morphological transformation and inhibition of growth of bovine luteal cells in tissue culture induced by luteinizing hormone and dibutyryl cyclic AMP. Endocrinology 1975; 96:458-467.

Grummer RR, Carrol DJ. A review of lipoprotein cholesterol metabolism: importance to ovarian function. J Anim Sci 1988; 66:3160-3173.

Gwynne JT, Strauss JF. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocrine Reviews 1982; 3:299-329.

Hafs HD, Armstrong DT. Corpus luteum growth and progesterone synthesis during the bovine estrous cycle. J Anim Sci 1968; 28:134-141.

Hall PF. Cellular organization for steroidogenesis. International Review of Cytology 1984; 46:53-95.

Hall PF, Koritz SB. Influence of interstitial cell-stimulating hormone on the conversion of cholesterol to progesterone by bovine corpus luteum. Biochemistry 1965; 4:1037-1043.

Hall PF. The role of the cytoskeleton in the supply of cholesterol for steroidogenesis. In: Strauss JF, Menon KMJ (ed.), Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues. Philadelphia, PA: George F Stickley Co; 1985:201-218.

Halterman SD, Murdoch WJ. Luteal function in ewes treated with antihistamines during the follicular phase. J Anim Sci 1986; 63(suppl 1):351 (abstract 467).

Hansel W, Concannon PW, Lukaszewska JH. Corpora lutea of the large domestic animals. Biol Reprod 1973; 8:222-225.

Hanson RW, Ballard FJ. The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. Biochem J 1976; 105:529-536.

Harrison LM, Randel RD. Influence of insulin and energy intake on ovulation rate, luteinizing hormone and progesterone in beef heifers. J Anim Sci 1986; 63:1228-

1235.

Hart IC, Bines JA, Morant SV, Ridley JL. Endocrine control of energy metabolism in the cow; comparison of the levels of hormones (prolactin, growth hormone, insulin, and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J Endocr 1978; 77:333-345.

Hay WW Jr, Sparks JW, Gilbert M, Battaglia FC, Meschia G. Effect of insulin on glucose uptake by the maternal hindlimb and uterus, and by the fetus in conscious pregnant sheep. J Endocr. 1984; 100:119-124.

Heider JG, Boyett RL. The picomole determination of free and total cholesterol in cells and culture. J Lipid Res 1978; 19:514-518.

Henderson KM, Moon YS. Luteinization of bovine granulosa cells and corpus luteum formation associated with loss of androgen-aromatizing ability. J Reprod Fert 1979; 56:89-97.

Hill JR Jr, Lamond DR, Henricks DM, Dickey JF, Niswender GD. The effects of undernutrition on ovarian function and fertility in beef heifers. Biol Reprod 1970; 2:78-84.

Hillers JK, Senger PL, Darlington RL, Fleming WN. Effects of production, season, age of cow, days dry, and days in milk on conception to first service in large commercial dairy herds. J Dairy Sci 1984; 67:861-867.

Hoffman B, Schams D, Bopp R, Ender ML, Gimenez T, Karg H. Luteotrophic factors in the cow: evidence for LH rather than prolactin. J Reprod Fert 1974; 40:77-85.

Hotta M, Baird A. The inhibition of low density lipoprotein metabolism by transforming growth factor-β mediates its effects on steroidogenesis in bovine adrenocortical cells *in vitro*. Endocrinology 1987; 121:150-159.

Hoyer PB, Fitz TA, Niswender GD. Hormone-independent activation of adenylate cyclase in large steroidogenic ovine luteal cells does not result in increased progesterone secretion. Endocrinology 1984; 114:604-608.

Hwang J, Menon KMJ. Characterization of low density and high density lipoprotein receptors in the rat corpus luteum and regulation by gonadotropin. J Biol Chem 1983; 258:8020-8027.

Iida S, Papadopoulos V, Hall PF. The influence of exogenous

free cholesterol on steroid synthesis in cultured adrenal cells. Endocrinology 1989; 124:2619-2624.

Iida S, Widmaier EP, Hall PF. The influence of plasma membrane cholesterol in the response of adrenal cells to adrenocorticotropin. Endocrinology 1987; 120:801-808.

Imakawa K, Day ML, Garcia-Winder M, Zalesky DD, Kittok RJ, Schanbacher BD, Kinder JE. Endocrine changes during restoration of estrous cycles following induction of anestrous by restricted nutrient intake in beef heifers. J Anim Sci 1986a; 63:565-571.

Imakawa K, Day ML, Zalesky DD, Garcia-Winder M, Kittok RJ, Kinder JE. Influence of dietary-induced weight changes on serum luteinizing hormone, estrogen and progesterone in the bovine female. Biol Reprod 1986b; 35:377-384.

Imakawa K, Kittok RJ, Kinder JE. Luteinizing hormone secretion after withdrawal of exogenous progestogen in heifers fed three levels of dietary energy. J Anim Sci 1984; 58:151-158.

Imakawa K, Kittok RJ, Kinder JE. The influence of dietary energy intake on progesterone concentrations in beef heifers. J Anim Sci 1983; 56:454-459.

Ingebritsen TS, Gibson DM. Reversible phosphorylation of hydroxymethylglutaryl CoA reductase. In: Cohen P (ed.), Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation. New York: Elsevier/North-Holland Biomedical Press; 1980:63-93.

Ingebrisen TS, Stewart AA, Cohen P. The protein phosphatases involved in cellular regulation. Eur J Biochem 1983: 132:297-307.

Ingle DL, Bauman DE, Garrigus US. Lipogenesis in the ruminant: *in vitro* study of tissue sites, carbon source and reducing equivalent generation for fatty acid synthesis. J Nutrition 1972; 102:609-616.

Ireland JJ, Roche JF. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroid in follicular fluid, and gonadotropin receptors. Endocrinology 1982; 111:2077-2086.

Johnson KR, Erb RE. Maintenance of pregnancy in ovariectomized cattle with progestin compounds and their effect on progestin levels in the corpus luteum. J Dairy Sci 1962; 45:631-639.

Kenny N, Farin CE, Niswender GD. Morphometric quantification of mitochondria in the two steroidogenic ovine luteal cell types. Biol Reprod 1989; 40:191-196.

Kimura M, Nakao T, Moriyoshim, Kawata K. Luteal phase deficiency as a possible cause of repeat breeding in dairy cows. Br Vet J 1987; 143:560-566.

King GJ. Normal, short and long postpartum estrous cycles in dairy and beef cows. Proc 10th Intl Congr Animal Reproduction and Artificial Insemination; 1984; Urbana-Champaign, IL. Pages 399-402.

Kittok RJ, Stellflug JN, Lowry SR. Enhanced progesterone and pregnancy rate after gonadotropin administration in lactating ewes. J Anim Sci 1983; 56:652-655.

Kliensik DA, Jabalquinto AM, Porter JW. *In vivo* and *in vitro* mechanisms regulating rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase Activity. J Biol Chem 1980; 255:3918-3923.

Koos RD, Hansel W. The large and small cells of the bovine corpus luteum: ultrastructural and functional differences. In: Schwartz NB, Hunziker-Dunn M (ed.), Dynamics of Ovarian Function. New York: Raven Press; 1981:197-203.

Koos RD. The potential relevance of angiogenic factors to ovarian physiology. Sem Reprod Endocrinol 1989; 7:29-40.

Koos RD, LeMarie WJ. Factors that may regulate the growth and regression of blood vessels in the ovary. Sem Reprod Endocrinol 1983; 1:295-307.

Kovanen PT, Basu SK, Goldstein JL, Brown MS. Low density lipoprotein receptors in bovine adrenal cortex. II. Low density lipoprotein binding to membranes prepared from fresh tissue. Endocrinology 1979; 104:610-616.

Kreiger M, Brown MS, Faust JR, Goldstein JL. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate. J Biol Chem 1978; 253:4093-4101.

Kris-Etherton PM, Etherton TD. The role of lipoproteins in lipid metabolism of meat animals. J Anim Sci 1982; 55:804-817.

Krishna A, Terranova PF. Alterations in mast cell degranulation and ovarian histamine in the proestrous hamster. Biol Reprod 1985; 32:1211-1217.

Landefeld TD, Campbell KL, Midgely, AR Jr. Rapid changes in the synthesis of specific ovarian granulosa cell proteins induced by human choriogonadotropin. Proc Natl Acad Sci 1979; 76:5153-5157.

Lange Y, Ramos BV. Analysis of the distribution of cholesterol in the intact cell. J Biol Chem 1983; 258:15130-15134.

Lange Y, Schmit VM, Schreiber, JR. Localization and movement of newly synthesized cholesterol in rat ovarian granulosa cells. Endocrinology 1988; 123:81-86.

Lehoux JG, Kandalaft N, Belisle S, Bellabarba D, Benard B, Lefebvre A. Increased 3-hydroxy-3-methyl-glutaryl coenzyme A reductase activity in an virilizing adrenal carcinoma. J Steroid Biochem 1984; 21:439-442.

Ling WY, Marsh JM. Reevaluation of the role of cyclic adenosine 3',5'-monophosphate and protein kinase in the stimulation of steroidogenesis by luteinizing hormone in bovine corpus luteum slices. Endocrinology 1977; 100:1571-1578.

Lino J, Baranao S, Hammond JH. Multihormone regulation of steroidogenesis in cultures porcine granulosa cells: studies in serum-free medium. Endocrinology 1985: 116:2143-2151.

Liscum L, Finer-Moore J, Stroud RM, Luskey KL, Brown MS, Goldstein JL. Domain structure of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a glycoprotein of the endoplasmic reticulum. J Biol Chem 1985; 260:522-530.

Lomax MA, Baird GD. Blood flow and nutrient exchange across the liver and gut of the dairy cow. Br J Nutr 1983; 49:481-496.

Lowry OH, Rosebrough NJ, Fair AL, Randall RJ. Protein measurements with the Folin-phenol reagent. J Biol Chem 1951; 193:265-275.

Luskey KL. Regulation of cholesterol synthesis: Mechanism for control of HMG-CoA Reductase. In: Clark JH (ed.), Recent Progress in Hormone Research, vol 44. San Diego, CA: Academic Press; 1988:35-51.

Marua T, Hayashi M, Matsuo H, Ueda Y, Morikawa H, Mochizuki M. Comparison of the facilitative roles of insulin and insulin-like growth factor I in the functional differentiation of granulosa cells: in vitro studies with the porcine model Acta Endocrinologica 1988;

117:230-240.

Mason NR, Savard K. Conversion of cholesterol to progesterone by corpus luteum slices. Endocrinology 1964; 75:215-221.

May JV, Schomberg DW. Developmental coordination of luteinizing hormone/human chorionic gonadotropin (hCG) receptors and acute hCG responsiveness in cultured and freshly harvested porcine granulosa cells. Endocrinology 1984; 114:153-163.

May JV, Schomberg DW. Granulosa cell differentiation in vitro: effect of insulin on growth and functional integrity. Biol Reprod 1981; 25:421-431.

McCann JP, Hansel W. Relationships between insulin and glucose metabolism and pituitary-ovarian functions in fasted heifers. Biol Reprod 1986; 34:630-641.

McGarry JD, Foster DW. Regulation of hepatic fatty acid oxidation and ketone body production. Ann Rev Biochem 1980; 49:395-420.

McIntosh EN, Uzgiris VI, Alonso C, Salhanick HA. Spectral properties, respiratory activity, and enzyme systems of bovine corpus luteum mitochondria. Biochemistry 1971; 10:2902-2923.

McLean MP, Miller JB. Indirect regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by oestradiol in the rabbit corpus luteum. J Reprod Fert 1988; 82:519-526.

Means AR, Chafouleas JG. Calmodulin in endocrine cells. In: Edelman IS (ed.) Ann Rev Physiol, vol 44. Palo Alto CA: Annual Reviews; 1982:667-682.

Mee MO, Stewart RE, Stevenson JS, Call EP. Prebreeding progesterone plus prostaglandin F_2-alpha influences estrus and fertility in lactating dairy cows. J Dairy Sci 1987; 70(suppl 1):205 (abstract p304).

Menon KMJ. Purification and properties of a protein kinase from bovine corpus luteum that is stimulated by cyclic adenosine 3′,5′-monophosphate and luteinizing hormone. J Biol Chem 1973; 248:494-501.

Milvae RA, Hansel W. Prostacyclin, prostaglandin $F_2\alpha$ and progesterone production by bovine luteal cells during the estrous cycle. Biol Reprod 1983; 29:1063-1068.

Mittre H, Aunai P, Benhaim A, Leymarie P. Acute stimulation by lutropin of mitochondrial protein synthesis in small

luteal cells. Eur J Biochem 1990; 187;721-726.

Murdoch WJ, Dunn TG. Alterations in follicular fluid steroid hormones during the preovulatory period in the ewe. Biol Reprod 1982; 27:300-307.

Nagy L, Freeman DA. Effect of cholesterol transport inhibitors on steroidogenesis and plasma membrane cholesterol transport in cultured MA-10 leydig tumor cells. Endocrinology 1990; 126:2267-2276.

Nakamura Y, Rhodes MT, Smith MF, Krishna A, Terranova PF. Increased number of mast cells in the dominant follicle of the cow: relationships among follicular, luteal, stromal and hilar regions. Biol Reprod 1987; 37:546-549.

National Research Council. Nutrient Requirements of Dairy Cattle, 5th rev ed. Washington, DC: National Academy of Sciences; 1978.

National Research Council. Nutrient Requirements of Dairy Cattle, 6th rev ed. Update. Washington, DC: National Academy of Sciences; 1989.

Nestle PJ, Poyser A, Hood RL, Mills SC, Willis MR, Cook LJ, Scott TW. The effect of dietary fat supplements on cholesterol metabolism in ruminants. J Lipid Res 1978; 19:899-909.

Nichikawa T, Mikami K, Saito Y, Tamura Y, Yoshida S. Functional differences in cholesterol ester hydrolase and acyl-coenzyme-A/cholesterol acyltransferase between the outer and inner zones of the guinea pig adrenal cortex. Endocrinology 1988; 122:877-883.

Niswender GD, Reimers TJ, Diekman MA, Nett TM. Blood flow: a mediator of ovarian function. Biol Reprod 1976; 14:64-81.

Noble RC. Digestion, absorption and transport of lipids in ruminant animals. In: Christie WW (ed.), Lipid Metabolism in Ruminant Animals, New York: Pergamon Press; 1981:57-94.

O'Shaughnessy PJ, Pearce S, Mannan MA. Effect of high-density lipoprotein on bovine granulosa cells: progesterone production in newly isolated cells and during cell culture. J Endocrinology 1990; 124:255-260.

O'Shaughnessy PJ, Wathes DC. Characteristics of bovine luteal cells in culture: morphology, proliferation and progesterone secretion in different media and effects of

LH, dibutyryl cyclic AMP, antioxidants and insulin. J Endocr 1985a; 104:355-361.

O'Shaughnessy PJ, Wathes DC. Role of lipoproteins and de-novo cholesterol synthesis in progesterone production by cultured bovine luteal cells. J Reprod Fert 1985b; 74:425-432.

O'Shea JD, Rodgers RJ, D'Occhio MJ. Cellular composition of the cyclic corpus luteum of the cow. J Reprod Fert 1989; 85:483-487.

Park CS, Rafalowski W, Marx GD. Effect of dietary fat supplement on lipid metabolism of Holstein heifers. J Dairy Sci 1983; 66:528-534.

Parmeggiani A, Bowman RH. Regulation of phosphofructokinase activity by citrate in normal and diabetic muscle. Biochem Biophys Res Comm 1963; 12:268-273.

Pate JL, Condon WA. Effects of serum and lipoproteins on sterlize Technical Soidogenesis in cultured bovine luteal cells. Mol Cell Endocrinol 1982; 28:551-562.

Pate JL, Condon WA. Regulation of steroidogenesis and cholesterol synthesis by prostaglandin F-2α and lipoproteins in bovine luteal cells. J Reprod Fert 1989; 87:439-446.

Pate JL, Nephew KP, Zarle GS. Cell density influences hormonal responsiveness but not lipoprotein utilization in cultured bovine luteal cells. Mol Cell Endocrinol 1987; 53:187-194.

Pederson RC, Brownie AC. Cholesterol side-chain cleavage in the rat adrenal cortex: isolation of a cyclohexamide-sensitive activator peptide. Proc Natl Acad Sci 1983; 80:1882-1886.

Pimental SM, Pimental CA, Weston PG, Hixon JE, Wagner WC. Progesterone secretion by the bovine fetoplacental unit and responsiveness of corpora lutea to steroidogenic stimuli at two stages of gestation. Am J Vet Res 1986; 47:1967-1977.

Poff JP, Fairchild DL, Condon WA. Effects of antibiotics and medium supplements on steroidogenesis in cultured cow luteal cells. J Reprod Fert 1988; 82:135-143.

Popewell PY, Azhar S. Effects of aging on cholesterol content and cholesterol-metabolizing enzymes in the rat adrenal gland. Endocrinology 1987; 121:64-73.

Popjak G, Clarke CF, Hadley C, Meenan A. Role of mevalonate in regulation of cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase in cultured cells and their cytoplasts. J Lipid Res 1985; 26:831-841.

Priedkalans J, Weber AF. Quantitative ultrastructural analysis of the follicular and luteal cells of the bovine ovary. Z Zellforsch 1968a; 91:574-585.

Priedlize Technical SAF. Ultrastructural studies of the bovine graafian follicle and corpus luteum. Z Zellforsch 1968b; 91:555-573.

Privalle CT, McNamara BC, Dhariwal MS, Jefcoate CR. ACTH control of cholesterol side-chain cleavage at adrenal mitochondrial cytochrome $P\text{-}450_{scc}$. Regulation of intramitochondrial cholesterol transfer. Mol Cell Endocrinol 1987; 53:87-101.

Proknor M, Dachir S, Owens RE, Little DE, Harms PG. Temporal relationship of the pulsatile fluctuation of luteinizing hormone and progesterone in cattle: a time series cross-correlation analysis. J Anim Sci 1986; 62:191-198.

Puppione DL. Implications of unique features of blood lipid transport in the lactating cow. J Dairy Sci 1978; 61:651-659.

Rahe CH, Owens RE, Fleeger JL, Newton HJ, Harms PG. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology 1980; 107:498-503.

Rainey WE, Shay JW, Mason JI. ACTH induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase, cholesterol biosynthesis and steroidogenesis in primary cultures of bovine adrenocortical cells. J Biol Chem 1986; 261:7322-7326.

Rajendran KG, Menon M, Peegel H, Hwang J, Menon KMJ. lize Technical Sma lipoproteins in steroidogenic response of rat luteal cells during gonadotropin-induced refractory states. Can J Physiol Pharmacol 1985; 63:265-272.

Rao CV, Estergreen VL, Carman FR Jr., Moss GE. Receptors for gonadotrophin and prostaglandin $F_2\alpha$ in bovine corpora lutea of early, mid and late luteal phase. Acta Endo 1979; 91:529-537.

Rao CV, Fields MJ, Chen TT, Abel JH Jr., Edgerton LA. Change in gonadotropin-binding sites in intracellular organelles and plasma membranes during luteal growth, development and regression. Experimental Cell Research 1983;

144:285-295.

Raphael BC, Dimick PS, Puppione DL. Lipid characterization of bovine serum lipoproteins throughout gestation and lactation. J Dairy Sci 1973; 56:1025-1032.

Rawn JD. Biochemistry. Burlington, NC: Neil Patterson Publishers; 1988:573.

Redmer DA, Grazul AT, Kirsch JD, Reynolds LP. Angiogenic activity of bovine corpora luteal at several stages of luteal development. J Reprod Fert 1988; 82:627-634.

Richards MW, Wettemann RP, Schoenemann HM. Nutritional anestrus in beef cows: Body weight change, body condition, luteinizing hormone in serum and ovarian activity. J Anim Sci 1989; 67:1520-1526.

Rinninger F, Pittman RC. Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters by human fibroblasts alize Technical Sma cells. J Lipid Res 1988; 29:1179-1194.

Rodgers RJ, Mason JI, Waterman, Simpson ER. Regulation of the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the bovine ovary *in vivo* and *in vitro*. Molecular Endocrinology 1987a; 1:172-180.

Rodgers RJ, Mitchell MD, Simpson ER. Secretion of progesterone and prostaglandins by cells of bovine corpora lutea from three stages of the luteal phase. J Endocr 1988; 118:121-126.

Rodgers RJ, Rodgers HF, Waterman MR, Simpson ER. Immunolocalization of cholesterol side-chain cleavage cytochrome P-450 and ultrastructural studies of bovine corpora lutea. J Reprod Fert 1986a; 78:639-652.

Rodgers RJ, O'Shea JD, Findlay JK. Do small and large luteal cells of the sheep interact in the production of progesterone? J Reprod Fert 1985; 75:85-94.

Rodgers RJ, Waterman MR, Simpson ER. Cytochromes P-450_{scc}, P-$450_{17\alpha}$, and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase in bovine follicles and corpora lutea. Changes in specific contents during the ovarian cycle. Endocrinology 1986b; 118:1366-1374.

Rodgers RJ, Waterman MR, Simpson ER. Levels of messenger ribonucleic acid encoding cholesterol side-chain cleavage cytochrome P-450, 17α-hydroxylase cytochrome P-450, adrenodoxin, and low density lipoprotein receptor in bovine follicles and corpora lutea throughout the ovarian

cycle. Molecular Endocrinology 1987b; 1:274-279.

Roser JF, Evans JW. Luteal luteinizing hormone receptors during the postovulatory period in the mare. Biol Reprod 1983; 29:499-510.

Rutter LM, Snopek R, Manns JG. Serum concentrations of IGF-I in postpartum beef cows. J Anim Sci 1989; 67:2060-2066.

SAS Institute Inc. SAS® User's Guide: Basics, version 5 Edition. Cary, NC: SAS Institute Inc; 1985a.

SAS Institute Inc. SAS® User's Guide: Statistics, version 5 Edition. Cary, NC: SAS Institute Inc; 1985b:113-138, 433-506, 655-710.

Savard K, Casey PJ. Effects of pituitary hormones and NADPH on acetate utilization in ovarian and adrenocortical tissues. Endocrinology 1964; 74:599-610.

Savard K, Marsh JM, Rice BF. Gonadotropins and ovarian steroidogenesis. In: Pincus G (ed.), Recent Progress in Hormone Research, vol 21, New York: Academic Press; 1965:285-365.

Savion N, Laherty,R, Cohen D, Lui GM, Gospodarowicz D. Role of lipoproteins and 3-hydroxy-3-methylglutaryl coenzyme A reductase in progesterone production by cultured bovine granulosa cells. Endocrinology 1982; 110:13-22.

Savion N, Laherty R, Lui G, Gospodarowicz D. Modulation of low density lipoprotein metabolism in bovine granulosa cells as a function of their steroidogenic activity. J Biol Chem 1981a: 24:12817-12822.

Savion N, Lui G, Laherty R, Gospodarowicz D. Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. Endocrinology 1981b; 109:409-420.

Scallen TJ, Vahouny GV. The participation of sterol carrier proteins in cholesterol biosynthesis, utilization and intracellular transfer. In: Strauss JF, Menon KMJ (ed.), Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues. Philadelphia, PA: George F Stickley Co; 1985:219-236.

Schaefer EJ, Eisenberg, Levy RI. Lipoprotein apoprotein metabolism. J Lipid Res 1978; 19:667-687.

Schams D, Koll R, Li CH. Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. J Endocr 1988; 116:97-100.

Schneider JE, Wade GN. Availability of metabolic fuels controls estrous cyclicity of Syrian hamsters. Science 1989; 244:1326-1328.

Schuler LA, Flickinger GL, Strauss JF. Effect of luteinizing hormone on the lipid composition of rat ovaries. J Endocr 1978; 78:233-238.

Schuler LA, Scavo L, Kirsch TM, Flickinger, Strauss JF. Regulation of *de novo* biosynthesis of cholesterol and progestins, and formation of cholesteryl ester in rat corpus luteum by exogenous sterol. J Biol Chem 1979; 254:8662-8668.

Schuler LA, Toaff ME, Strauss JF. Regulation of ovarian cholesterol metabolism: control of 3-hydroxy-3-methylglutaryl coenzyme A reductase and acyl coenzyme A:cholesterol acyltransferase. Endocrinology 1981; 108:1476-1486.

Schwall RH, Gamboni F, Mayan MH, Niswender GD. Changes in the distrubution of sizes of ovine luteal cells during the estrous cycle. Biol Reprod 1986; 34:911-918.

Silavin SL, Moss GE, Niswender GD. Regulation of steroidogenesis in the ovine corpus luteum. Steroids 1980; 36:229-241.

Silivan SL, Strauss JF. Progesterone production by hamster granulosa and luteal cells during short-term incubation. Effects of lipoproteins, compactin and 25-hydroxycholesterol. Biol Reprod 1983; 29:1163-1171.

Smith MF. Recent advances in corpus luteum physiology. J Dairy Sci 1986; 69:911-926.

Smith RG, Sestili MA. Methods for ligand-receptor assays in clinical chemistry. Clin Chem 1980; 26:543-550.

Snook RB, Brunner MA, Saatman RR, Hansel W. The effect of antisera to bovine LH in hysterectomized and intact heifers. Biol Reprod 1969; 1:49-58.

Soto EA, Tureck RW, Strauss JF. Human chorionic gonadotropin regulates metabolism of low density lipoproteins by human luteinized granulosa cells. In: Strauss JF, Menon KMJ (ed.), Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues. Philadelphia, PA: George F Stickley Co; 1985:59-68.

Spicer LJ, Ireland JJ, Roche JF. Changes in serum LH, progesterone, and specific binding of ^{125}I-hCG to luteal cells during regression and development of bovine corpora

lutea. Biol Reprod 1981; 23:832-841.

Spitzer JC, Niswender, Seidel, GE Jr, Wiltbank JN. Fertilization and blood levels ofprogesterone and LH in beef heifers on a restricted diet. J Anim Sci 1978; 46:1071-1077.

Sreenan JM, Diskin MG. Early embryonic mortality in the cow: its relationship with progesterone concentration. Veterinary Record 1983; 112:517-521.

Stewart RE, Mee MO, Stevenson JS. Concentrations of progesterone and conception rates in Holstein heifers after human chorionic gonadotropin (hCG) administration during the first 3 weeks after estrus. J Anim Sci 1987; 65(suppl 1):376 (abstract 434).

Storry JE, Brumby PE, Tuckley B, Welch VA, Stead D, Fulford RJ. Effects of feeding protected lipid to dairy cows in early lactation on the composition of blood lipoproteins and secretion of fatty acids in milk. J Agric Sci, Camb 1980; 94:503-516.

Stranberg TE, Tilves RS. Physiological and pharmacological regulation of small intestinal cholesterol synthesis. Gen Pharmac 1988; 19:321-329.

Strauss JF, Schuler LA, Rosenblum MF, Tanaka T. Cholesterol metabolism by ovarian tissue. Advances in Lipid Research 1981; 18:99-157.

Suckling KE, Stange EF. Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. J Lipid Res 1985; 26:647-671.

Swann RT, Bruce NW. Acetate and plasma cholesterol as progesterone precursors in the intact ovary of the Day-16 pregnant rat. J Reprod Fert 1986; 77:655-664.

Talvera F. Park CS, Williams GL. Relationships among dietary lipid intake, serum cholesterol and ovarian function in Holstein heifers. J Anim Sci 1985; 60:1045-1051.

Tan CH, Robinson J. The superovulated rat. Its use as a model in studies on the acute steroidogenic effects of luteinizing hormone. Endocrinology 1977; 101:396-402.

Teranova PF, Byrd C. Shift in capillarity of ovarian follicles during an LH-induced superovulation in cyclic hamsters. Biol Reprod 1986; 34(suppl 1):210 (abstract 322).

Tharp MD. The interaction between mast cells and endothelial

cells. J Invest Dermatol 1989; 93:107S-112S.

Thatcher WW, Larson LE Jr, Drost M, Putney DJ. HCG-induced alterations in pregnancy rate of lactating dairy cows during summer months in south Florida. J Dairy Sci 1987; 70(Suppl 1):206 (abstract p307).

Trzeciak WH, Simpson ER, Scallen TJ, Vahouny GV, Waterman MR. Studies on the synthesis of sterol carrier protein-2 in rat adrenocortical cells in monolayer culture. J Biol Chem 1987; 262:3713-3717.

Trzeciak WH, Waterman MR, Simpson ER. Synthesis of the cholesterol side-chain cleavage enzymes in cultured rat ovarian granulosa cells: induction by follicle-stimulating hormone and dibutyryl adenosine 3′,5′-monophosphate. Endocrinology 1986; 119:323-330.

Tuckey RC, Stevenson PM. Cholesteryl esterase and endogenous cholesteryl ester pools in ovaries from maturing and superovulated immature rats. Biochim Biophys Acta 1980; 618:501-509.

Ursely J, Leymarie P. Varying response to luteinizing hormone of two luteal cell type isolated from bovine corpus luteum. J Endocrinol 1979; 83:303-310.

Uzgiris VI, McIntosh EN, Alonso C, Salhanick HA. Role of reversed electron transport in bovine corpus luteum mitochondrial steroid synthesis. Biochemistry 1971; 10:2916-2923.

Veldhuis JD, Gwynne JT. Properties of low density lipoprotein binding by cultured swine granulosa cells. Endocrinology 1985a; 117:1067-1074.

Veldhuis JD, Nestler JE, Strauss JF. Insulin regulates low density lipoprotein metabolism by swine granulosa cells. Endocrinology 1986; 118:2242-2253.

Veldhuis JD, Nestler JE, Strauss JF. The insulin-like growth factor, somatomedin-C, modulates low density lipoprotein metabolism by swine granulosa cells. Endocrinology 1987; 121:340-346.

Veldhuis JD, Strauss JF, Silivin SL, Kolp LA. The role of cholesterol esterification in ovarian steroidogenesis: studies in cultured swine granulosa cells using a novel inhibitor of acyl coenzyme A:Cholesterol acyltransferase. Endocrinology 1985b; 116:25-30.

Vernon RC, Finley E, Taylor E, Flint DJ. Insulin binding and action on bovine adipocytes. Endocrinology 1985;

116:1195-1199.

Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL. Association between energy balance and luteal function in lactating dairy cows. J Dairy Sci 1988; 71:1063-1072.

Villa-Godoy A, Hughes TL, Emery RS, Enright WJ, Ealy AD, Zinn SA, Fogwell RL. Energy balance and body condition influence luteal function of Holstein heifers. Domestic Aniaml Endocrinology 1990; 7:135-148.

Walters DL, Schams D, Schallenberger E. Pulsatile secretion of gonadotropins, ovarian steroids and ovarian oxytocin during the luteal phase of the oestrous cycle in the cow. J Reprod Fert 1984; 71:479-491.

Williams GL. Modulation of luteal activity in postpartum beef cows through changes in dietary lipid. J Anim Sci 1989; 67:785-793.

Williams MT, Clark MR, Ling WY, LeMarie WJ, Gibson, MG, Marsh, JM. Role of cyclic AMP in the actions of luteinizing hormone on steroidogenesis in the corpus luteum. In: George WJ, Ignarro LJ (ed.), Advances in Cyclic Nucleotide Research. New York: Raven Press; 1978:573-582.

Williams MT, Marsh JM. Cytochalasin B inhibition of luteinizing hormone action on bovine luteal cells. In: Channing CP, Marsh JM, Sadler WA (ed.), Ovarian Follicular and Corpus Luteum Function. New York: Plenum Publishers; 1979:549-554.

Wise TH, Caton D, Thatcher WW, Barron DH, Fields MJ. Ovarian function during the estrous cycle of the cow: ovarian blood flow and progesterone release rate. J Anim Sci 1982; 55:627-637.

Yanagibashi K, Ohono Y, Kawamura M, Hall PF. The regulation of intracellular transport of cholesterol in bovine adrenal cells: purification of a novel protein. Endocrinology 1988; 123:2075-2082.

Youdan PG, King JOL. The effects of body weight changes on fertility during the post-partum period in dairy cows. Br Vet J 1977; 133:635-641.

Zammit VA, Easom RA. Regulation of hepatic HMG-CoA reductase in vivo by reversible phosphorylation. Biochim Biphys Acta 1987; 927:223-228.