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STUDIES ON OVINE PLACENTAL LACTOGEN

by

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To

My parents and wife:

Mr. & Mrs. Kai-On Chan

and

Mrs. Ting-Mei Chan

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CONTENTS

LIST OF ABBREVIATION

Peptide Hormones:

ACTH	Adrenocorticotropic hormone
CG	Chorionic Gonadotropin
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
LH	Luteinizing Hormone
PRL	Prolactin
PL	Placental Lactogen

Prefix to hormones:

b	bovine
h	human
o	ovine
r	rat
c	caprine
mou	mouse
m	monkey
gp	guinea pig
ham and p	hamster and porcine

Others:

C	Degree Centigrade
cpm	Counts per minute
g	Gram
mg	Milligram
ug	Microgram

M	Molar
Mm	Millimolar
ul	Microliter
N	Normal
ng	Nanogram
l	Liter
g	Gravitational force
IU	International unit
U	Unit

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ABSTRACT

STUDIES ON OVINE PLACENTAL LACTOGEN (oPL). John S.D. Chan, Dept. of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Using conventional protein purification procedures and radioreceptor assay for growth hormone (RRA-GH), oPL was purified to near homogeneity (greater than 2,000-fold) from ovine placental cotyledons. The molecular weight of oPL is approximately 21,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Specific binding sites for oPL have been found in ovine liver (maternal and fetal), adipose tissue, ovary, corpus luteum, and non-pregnant uterus. Specific studies showed that only growth hormone preparations could displace oPL binding to its receptors whereas other hormone preparations could not, indicating that oPL binds specifically to GH-receptor sites in ovine tissues. By employing the radioreceptor assay for placental lactogen (PL) using oPL and ovine liver as hormone standard and receptor respectively, PL-like activity was detected in placental extracts of human, monkey, goat, guinea pig, and mouse but not in cow, pig, horse, dog, rat, and rabbit. Homologous radioimmunoassay (RIA) for oPL has been developed in which ovine pituitary prolactin (oPRL) and growth hormone (oGH) as well as other pituitary and placental hormones from several species exhibit no cross-reaction. Using this RIA, oPL is detectable in the uterine vein blood samples as early as 26 days of gestation.

The secretory pattern of oPL in the maternal circulation is similar to that of human and monkey placental lactogen with the peak level in both circulation and placentomes after 80 days of gestation. oPL is found in the fetal circulation and allantoic fluids throughout pregnancy with high levels during early pregnancy. oPL is not detectable in amniotic fluid and maternal urine. oPL is dectable in extracts of chorionic membranes and maternal caruncles as early as 16 days of gestation with high concentration in fetal membranes. These studies indicate that oPL is secreted from fetal tissues. This is supported by the biosynthesis studies which showed that oPL is synthesized and secreted by chorio-allantoic membranes as early as 22 days of pregnancy. The half-time disappearance rate ($t_{\frac{1}{2}}$) of oPL in sheep is approximately 60 min. When oPRL and oGH were measured by specific RIAs in maternal blood samples during pregnancy, the levels of oPRL are inversely related to those of oPL without any major variation in oGH levels. This finding suggests that oPL might be the hormone that suppresses oPRL secretion during pregnancy. In the bioassay using hypophysectomized rat tibial width increase as an index of somatogenic effects, oPL is 1.5 times more potent than a bovine GH (1.0 U/mg) standard. In pseudopregnant rats, administration of oPL into the animals can prevent the loss of LH-receptors in the corpora lutea and the fall of progesterone after PGF_{2a} or bromoergocryptine (CB-154) injection. These studies suggest that during pregnancy oPL may act as a "growth hormone" of pregnancy and that it helps to maintain the integrity of LH-receptors in the corpora lutea. Finally,

receptor-binding studies show that only oPL and human growth hormone (hGH) bind to animal and human tissue receptors whereas other growth hormone preparations from other species do not. These studies suggest that the binding sites in oPL and hGH are very similar in conformation. Thus, further structural analysis on the active sites for binding and for growth promoting activity may have potential implication for future clinical use.

SECTION 1 REVIEW OF PLACENTAL PROTEIN AND POLYPEPTIDE HORMONES

A. INTRODUCTION:

The importance of the placenta as an active participant in providing for an intrauterine milieu favorable for fetal survival has been surmised since antiquity. Modern investigative techniques have, however, removed this subject from the area of conjecture and provided understanding of the mechanisms by which placental tissue carries out some of its functions. One of the best known capabilities of this highly developed, though transitory, tissue is the maintenance of fetal-maternal gradients in terms of gases and metabolites which are favorable to the fetus. Less well understood, however, is the role played by the placenta as an endocrine organ. Study of the secretions of placental tissue has been hindered by several problems which were not encountered in the classical studies of the pituitary and its target organs. The method of total extirpation and replacement treatment, for example, is denied the investigator of placental endocrinology. Furthermore, as our knowledge of the endocrine functions of the placenta has grown it is becoming apparent that, unlike the secretions of the anterior pituitary, the secretions of the placenta vary widely from one species to another. Variation is also apparent within a given species at different stages of gestation. Such problems were not to become a prohibitive barrier to investigation, however, and recent

years have seen an increasing interest in this facet of reproductive physiology.

The placenta of the human, as well as its fetus, is known to participate in a variety of steriod synthetic reactions as reviewed by several investigators (Diczfalusy 1968; Solomon and Friesen 1968; Villee 1969). The present review describes only the placental peptide hormones of those few animal species in which such secretions have been studied. Particular emphasis will be placed on primates, since considerable amount of data has been made in these species.

B. BRIEF SUMMARY ON THE EARLY INVESTIGATIONS OF THE ENDOCRINE PLACENTA:

The capacity of the placenta to act as an endocrine organ was first postulated by Bouchacourt (1903) based on clinical evidence that he was successful in stimulating lactation in women with oral doses of "chorinine", an extract of sow placenta. At about the same time Halban (1905) found that ovariectomy did not lead to termination of pregnancy in humans, suggesting that the placenta is capable of taking over the function of the ovary during pregnancy. Moreover, Starling also found that extracts prepared from the rabbit fetus produced mammary gland growth when injected into virgin female rabbits (Starling 1905). Both were agreed that mammary development could occur independently of the ovaries. Later in 1913 Aschner (Aschner 1913) also reported that placental

extracts have luteotropic effects when injected into lower animals. These and other early events in the study of the placenta as an endocrine gland have been reviewed by several investigators (Lyons 1958; Deansely 1966; Simmer 1968).

C. COMPARATIVE ASSESSMENT OF PLACENTAL PROTEIN HORMONES PRODUCTION IN SEVERAL ORDERS OF MAMMALS:

1. PRIMATE AND SUBPRIMATE PLACENTAL PROTEIN HORMONES

Ia PRIMATE (HUMAN)

A. HUMAN CHORIONIC GONADOTROPIN (hCG)

i) INTRODUCTION:

In the two decades following the publication of Halban's view on the endocrine activities of placental extracts, no significant progress in this field was made. The next important landmark in the history of the study of placental endocrinology was the announcement in 1927 by Ascheim and Zondek (Ascheim 1927) that the urine of pregnant women contained a potent gonadotropin capable of producing follicular growth and luteinization in the immature mouse ovary. Several years later, Selye et al (1933) reported that in hypophysectomized rats the endocrine function of the pituitary gland was not necessary for the continuation of normal pregnancy and parturition. Subsequently studies by Evans and coworkers (Evans et al 1933, 1935) demonstrated that urinary gonadotropins were found in patients with embryonic neoplasia. Their findings lent support to the concept of placental

secretion of a peptide hormone.

Thus, the first placental protein hormone to be recognized and characterized to some degree was human chorionic gonadotropin (hCG). Initially in the 1930s, it was called "prolan" because of its availability in copious quantities from urine of pregnant women. It now has been studied in terms of its chemistry, physiology, and immunological properties.

ii) CHEMISTRY OF hCG:

hCG is a glycoprotein of 39,000 molecular weight (Got et al, 1960; Bahl et al, 1972), about 30% of its weight is carbohydrate, with N-acetylneuraminic acid (NANA, sialic acid) and fucose as nonreducing terminal units (Bahl, 1969a). The high sialic acid content accounts for its low isoelectric point of 2.95. Variations in sialic acid content have been claimed to account for electrophoretic heterogeneity seen also on isoelectric focusing (Bell et al, 1969; Weise et al, 1973).

Structural studies made on hCG obtained from human pregnancy urine show it to consist of two dissimilar, non-covalently-bound subunits alpha and beta, which can be dissociated by urea and have been isolated and purified (Swaminathan and Bahl, 1970; Canfield et al, 1970, 1971; Rathnam and Saxena, 1972). A striking feature of the amino acid composition of hCG is its high content of proline, second only to that of collagen, indicative of a low alpha-helical content consistent with the circular dichroic spectra of the molecule (Hilgenfelt et al, 1974). A large amount of serine is also present,

whereas tryptophan is absent.

a) The Alpha-Subunit:

The alpha subunit of hCG is a glycopeptide with a molecular weight of 14,900, of which the peptide portion represents 10,200 and the carbohydrate 4,700. The complete amino acid sequence of 89-92 amino acid residues for hCG-alpha has been proposed by Morgan and coworkers (1972, 1975) and by Bahl and associates (Bahl, 1972b; Bahl et al, 1972; Belisario et al, 1973). It is shown in Figure 1. Both groups of workers found heterogeneity at the amino terminus. Approximately 30% of all hCG-alpha chains lacked the terminal tripeptide, and another 10% the terminal dipeptide. Such heterogeneity could represent precursor molecules at various stages of cleavage but could arise from digestion during urinary excretion or even during purification of the hormone. hCG-alpha has five disulfide bonds, but there is no evidence of free sulhydryl groups. The assignment of the disulfide bonds as well as of some of the amino groups still remains to be completed.

The total carbohydrate of hCG-alpha is contained in two bulky branched oligosaccharide side chains attached by N-glycosidic bonds to asparagine residues at positions 52 and 78. The chains terminate in N-acetylneuraminic acid and have galactosyl residues in the immediate subterminal positions (Bahl, 1972b). The amino acid sequences at the sites of attachment are of the type Asn-X-Thr commonly

NH_2-Ala-Pro-Asp-Val-Gln-Asp-Cys-Pro-Glu-Cys-Thr-Leu-Gln-Glu-Asp-
1 5 10 15

Pro-Phe-Phe-Ser-Gln-Pro-Gly-Ala-Pro-Ile-Leu-Gln-Cys-Met-Gly-
20 25 30

Cys-Cys-Phe-Ser-Arg-Ala-Tyr-Pro-Thr-Pro-Leu-Arg-Ser-Lys-Lys-
35 40 45

Thr-Met-Leu-Val-Gln-Lys-Asn-Val-Thr-Ser-Glu-Ser-Thr-Cys-Cys-
50 55 60

Val-Ala-Lys-Ser-Tyr-Asn-Arg-Val-Thr-Val-Met-Gly-Gly-Phe-Lys-
65 70 75

Val-Glu-Asn-His-Thr-Ala-Cys-His-Cys-Ser-Thr-Cys-Tyr-Tyr-His-
80 85 90

Lys-Ser-COOH

Figure 1.Linear sequence of amino acids in the α-subunit of Human chorionic gonadotropin. Based on Morgan et al (1975)

associated with carbohydrate linkages.

b) The Beta-subunit:

The molecular weight of hCG-beta is approximately 23,000, some 16,000 is accounted for by the peptide portion and 7,000 by the carbohydrate portion of the molecule. The linear amino acid sequence has been proposed by Morgan et al (1975), Bahl et al (1972), and Carlsen et al (1973). It is shown in Figure 2. There are, however, several discrepancies between the proposed structures. Canfield's group has reported 145 amino acids present whereas Bahl and coworkers have found 147 a.a. residues. Neither group has reported any N- or C- terminal heterogeneity.

In addition, these investigators differ as to the number and positions of carbohydrate attachments to the hCG-beta subunit. Both groups of investigators reported 12-cysteine residues (Swaminathan and Bahl, 1970; Morgan and Canfield, 1971). However, the positions of the 6 disulfide briges have yet to be assigned. As in hCG-alpha, no free sulfhydryl groups have been detected, and early reports (Bahl, 1969a; Bell et al, 1969) of the presence of tryptophan were not confirmed when the final sequence analysis was completed.

hCG-beta has an additional 30 residues at the C-terminal that are not found in any of the other glycoprotein hormone beta subunits. These additional residues may account for the biological and immunological specificity of hCG-beta. Nine of these residues are prolines, and 4 of the serines

NH_2-Ser-Lys-Glu-Pro-Leu-Arg-Pro-Arg-Cys-Arg-Pro-Ile-Asn-Ala-Thr-
1 5 10 15

Leu-ALa-Val-Glu-Lys-Glu-Gly-Cys-Pro-Val-Cys-Ile-Thr-Val-Asn-
20 25 30

Thr-Thr-Ile-Cys-Ala-Gly-Tyr-Cys-Pro-Thr-Met-Thr-Arg-Val-Leu-
35 40 45

Gln-Gly-Val-Leu-Pro-Ala-Leu-Pro-Gln-Val-Val-Cys-Asn-Tyr-Arg-
50 55 60

Asp-Val-Arg-Phe-Glu-Ser-Ile-Arg-Leu-Pro-Gly-Cys-Pro-Arg-Gly-
65 70 75

Val-Asn-Pro-Val-Val-Ser-Tyr-Ala-Val-Ala-Leu-Ser-Cys-Gln-Cys-
80 85 90

Ala-Leu-Cys-Arg-Arg-Ser-Thr-Thr-Asp-Cys-Gly-Gly-Pro-Lys-Asp-
95 100 105

His-Pro-Leu-Thr-Cys-Asp-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-
110 115 120

Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-
125 130 135

Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln-COOH
140 145

Figure 2. Amino acid sequence of the β chain of human chorionic gonadotropin. Adapted and modified from Morgan et al (1975)

possess carbohydrate side chains. This region is quite resistant to proteolysis in vitro and may protect the molecule from proteolytic cleavage in vivo, possibly accounting for the longer circulating half-life of hCG than of the other glycoprotein hormones.

It has been reported that rabbit anti-hCG beta can discriminate immunologically between hCG and hLH (Ross et al, 1972; Vaitukaitis et al, 1972b). These antibodies probably recognize the unique structural feature at the C terminus of hCG-beta. Recently, Schneider et al (1975) have reported the chemical synthesis of an eicosapeptide related to the C-terminus of hCG-beta, which is capable of inducing the formation of antibodies that can interact with the entire hCG molecule. This specific sequence has been used to prepare antibodies for fertility control (Talwar et al, 1976). hCG has also been reported to have FSH-like activity (Albert, 1969), and it is possible that these terminal residues (which comprise the major difference between hCG and hLH) are responsible for this alleged activity.

c) Structure-Activity Relationship:

Two types of structural modifications, namely subunit dissociation and removal of sugar residues, have been shown to affect the biological and immunological activity of hCG.

a- Dissociation of Subunits-

Treatment with urea dissociates hCG into its subunits, which are devoid of biological activity (Morgan and Canfield,

1971; Rayford et al, 1972). Consistent with this, no ovarian uptake of tritium-labeled alpha or beta subunits is found in vivo (Braunstein et al, 1972b) and neither subunit significantly inhibits hCG in vitro (Lee and Ryan, 1972; Catt et al, 1973). However, the dissociated subunits can be recombined when incubated in equimolar concentrations in solution to form native hCG with the same electrophoretic mobility and 80% of the original biological activity (Canfield et al, 1971; Morgan et al, 1971; Pierce et al, 1971; Bahla, 1972a; Rathnam et al, 1972; Morgan et al, 1974). Two possible reasons for the lack of in vivo biological activity of the isolated subunits are that they do no survive long enough to reach their target cells and that they bind poorly to receptors on target cell membranes. In connection with the former, Braunstein et al. (1972b) report a much shorter half-life for dissociated hCG subunits compared with the undissociated hormone when injected into female rats. The initial half-life of hCG-alpha in the plasma was 6 minutes, hCG-beta was 11 minutes, and the half-life of intact hCG was 141 minutes. In addition to shortened survival, Kammerman et al. (1972) found that the subunits did not bind to target cells in vitro.

b- Removal of Carbohydrates-

The biological specific activity of hCG preparations decreases with progressive removal of sialic acid, notably on loss of the terminal sialic acid residues in the molecule (Goverde et al., 1968; Van Hell et al, 1970; Braunstein et al, 1971; Brossmer et al, 1971; Graesslin et al, 1972) with less

than 1% activity remaining after removal of 70% of the total sialic acid residues (Van Hall et al, 1971a). The decrease in biological activity is accompanied by a decrease in the plasma half-life of the desialylated hormone. It is suggested that a role for the sialic acid of hCG is the protection of the hormone against metabolic degradation. Desialylation does take place in vivo, since extracts of urine from pregnant women contain variably desialylated hCG molecules (Goverde et al, 1968).

Bahl and Merz (1974) report that removal of sialic acid results in a two-fold increase in the binding of hCG to specific plasma membrane receptors in vitro. In an assay system based upon stimulation of testosterone secretion in rat Leydig cells in vitro, completely desialylated hCG retained about 25% of the activity of the native hormone (Dufau et al, 1971). Similarly, desialylated hCG only partially inhibited the binding of the hormone to ovarian tissue (Tsuruhara et al, 1972a). However, Moyle et al (1973) found removal of sialic acid caused an 80% loss in the ability of gonadotropins to stimulate adenyl cyclase in suspensions of rat Leydig cells. These studies suggest that sialic acid is not required for the recognition of the hormone by receptor, hormone-receptor-interaction, or target-organ stimulation, but is required for biological response.

In contrast, the immunological activity of hCG is not affected by removal of over 95% of the sialic acid (Van Hall et al, 1971a; Merz et al, 1974a), and the removal of

sialic acid did not alter the complement-fixing activity of hCG (Mori, 1970).

c- Other Modifications-

Tyrosine residues are thought to be important to protein structure and activity. A recent study employing the reaction of hCG with tetranitromethane (Hum et al, 1974) suggests that Tyrosine-88 and tyrosine-89 in the hCG-alpha subunit are important to the biological activity of hCG, but not the tyrosine residues of the beta-subunit (at positions 58 and 81), and that the Tyrosine-65 of the alpha is involved in holding the alpha and beta subunits together in native conformation.

Finally, there is a report of trypsin digestion of hCG down to a core of 6000 molecular weight, which still retains biological activity (Kikutani et al, 1965), similar to the claim made for FSH digested with papain (Rathnam and Saxena, 1970).

iii) CELL OF ORIGIN AND BIOSYNTHESIS OF hCG:

a- Cell of orgin of hCG

Although early histochemical studies suggested secretion of hCG by both cytotrophoblast and syncytiotrophoblast, the advent of immunohistochemistry has provided more precise evidence of its origin. Recent studies (Bossart et al, 1965; Dreskin et al, 1970; Ikonikoff et al, 1973) using peroxidase-labelled antibody to hCG have shown that this hormone is primarily located in the syncytiotrophoblast of both early

and term placentas. This identification agrees with the presence of well-developed rough endoplasmic reticulum in the syncytiotrophoblast, but not in the cytotrophoblast cells. In the case of trophoblast tumors which remain discretely cellular, Gartner et al (1975) have nevertheless found hCG by immunohistochemical means in cells that had not fully passed from cytotrophoblast to syncytiotrophoblast. Regarding the details of secretion of hCG by the syncytiotrophoblast, studies combining antibody binding with electron microscopy have shown that hCG is present on the maternal surface of the apical plasma membrane, in the cisterna of the rough endoplasmic reticulum of the syncytiotrophoblast, and on the outer surface of the basal plasma membrane (Dreskin et al, 1970; Genbacev et al, 1975) suggesting secretion into both the maternal and fetal circulations. None was found in the lamellae and vesicles of the Golgi apparatus or in the cytotrophoblast.

b- Biosynthesis of hCG-

Direct proof that the placenta synthesizes hCG was first obtained by Kito (1937), who transplanted chorionic tissue into the anterior chamber of the eye of a rabbit and demonstrated hCG-like effects on the host's ovaries as well as the secretion of large amounts of the biologically active hCG in the animal's urine. This experiment has been repeated and confirmed by others. More recently, there have been a few studies of hCG synthesis by placental tissue slices incubated in vitro with labelled amino acids. Gitlin

and Biasucci (1969) demonstrated synthesis of hCG by incubating placental tissue in a ^{14}C-labelled amino acid mixture and submitting the medium after incubation to immunoelectrophoresis and autoradiography. They established that hCG synthesis was greater in placentas from 4 to 10 weeks gestation than in 14 to 18 week placentas.

The mechanism of synthesis of the peptide portion of hCG has been explored using cell-free systems in which polyribosomes carrying nascent peptide chains or mRNA extracted from these are translated. Chatterjee et al (1976 and 1977) have examined the amount of hCG synthesized by the first trimester and term polyribosomes prepared from human placentas. The polyribosomes were incubated with tritium-leucine, and the labelled hCG chains were precipitated with carrier hCG and anti-hCG serum. The immunoprecipitate was resolved on SDS gels, which were then cut into small slices. They demonstrated that there are two partially resolved radio-active peaks corresponding to peptides of MW 10,000 and 16,000, which are the molecular weights of the alpha and beta chains of hCG before addition of sugars. The identity of these two peaks were confirmed by subsequent experiments in which specific antisera for each subunit of hCG was used.

This technique was used to show that hCG peptides are predominantly synthesized on membrane-attached ribosomes, in conformity with the pattern for the secreted protein (Munro et al, 1974). Polyribosomes prepared from first-trimester placenta showed 11% of all nascent peptides to be hCG, whereas

by term the percentage had fallen to 2% (Chatterjee et al, 1976). This implied that the amount of hCG made by the placenta, and thus the amount present in maternal plasma, is determined by availability of specific mRNA. Furthermore, when Chatterjee (1977) measured the synthesis of alpha and beta chains by separate immunoprecipitation at two stages of pregnancy (first trimester and term), they showed that the amount of the alpha-chain on the polyribosomes declines from 4.9 to 1.3% of total peptide synthesis, whereas synthesis of the beta-subunit falls from 3.9% to 0.6%. Thus the ratio of alpha to beta chain radioactivity increases from 1.2 in the first trimester to 2.3 at term. This coincides with the presence of increasing amounts of free alpha-chains in the plasma as pregnancy progresses, and demonstrates that production of the two chains is not necessarily coordinated and that the amount of hCG secreted into the plsama is limited by the availability of the beta-chain.

iv) Regulation of Secretion of hCG (in vitro):

Regulation of secretion of hCG has been studied in placental slices and in hormone-secreting cells in culture. hCG is rapidly released from placental slices after synthesis, as judged by kinetics of appearance in the incubation media of peptides containing labelled precursor amino acids (Patrito et al, 1973; Maruo et al, 1974; Tojo et al, 1974).

Several attempts have been made to determine subcellular factors regulating synthesis of hCG. Synthesis of hCG by trophoblastic cells in culture is stimulated by

addition of cAMP (Handwerger et al, 1973; Hussa et al, 1974; Story et al, 1974). Furthermore, Bolander et al (1978) using culture explants from near term placental tissue showed that the secretion of hCG into the medium increased 7-8 fold during the second to forth day of incubation, suggesting that the synthesis of hCG from the term placenta is subject to an inhibitory influence which declines with time in culture.

A new and significant factor in the control of hCG secretion may have emerged with the claim to have identified luteotropin-releasing factor (LRF) activity in the placenta (Gilbons et al, 1975). Since LRF secreted by the hypothalamus regulated output of the corresonding gonadotropin LH from the pituitary gland, this observation suggests that control of hCG may occur through LRF made in the placenta, possibly in the cytotrophoblast. There, cAMP would then be formed and cause hCG output to be stimulated. This theory is compatible with the synthesis of hCG by the syncytiotrophoblast, but not cytotrophoblast, and the increased release of hCG by cAMP noted above for cells in culture. The formation of LRF may decline in later pregnancy and explain the decline in hCG formation. Although Gilboms's hypothesis is interesting, there are no further studies to confirm his claims.

v) Secretion and Metabolism of hCG:

Using a variety of biological and immunological assay procedures, numerous investigators have reported on the concentrations of hCG in the urine and serum of pregnant

women. The hormone is present in blood and urine as early as 9 to 12 days after fertilization (Kousa et al, 1974; Catt et al, 1975). The hCG concentrations rise rapidly and peak levels occur at around 7-10 weeks after the last menstrual period, when it attains about 2.5-5 μg (50-100 IU) per milliter of serum. By midpregnancy the level has declined to a mean of less than 0.5 μg (10 IU)/ml and then remains at a constant low level until term. The rate of hCG production has been calculated to be about 26 mg/day at peak levels and 1.4 mg/day during the latter half of gestation (Rizkallah et al, 1969).

In multiple pregnancies, hCG levels are reported to be elevated (Halgin, 1970), perhaps owing to the increased placental mass. The abnormally high plasma hCG levels of diabetic pregnancies are also associated with large placentas and fetuses (Samaan et al, 1969). There is also a difference in serum hCG levels and placental hCG content for male and for female fetuses during the third trimester, that for the males being lower (Brody and Carlstrom, 1965b; Crosignani et al, 1972; Hobson et al, 1974; Penny et al, 1974; Boroditsky et al, 1975), although Spellacy et al (1975) failed to observe a difference in serum level. hCG is also found in the amniotic fluid at levels lower than, but proportional to, the maternal level of hCG (Berle, 1969). The hCG concentration in infants at birth is approx. 300 mIU/ml. Turnover of hCG has been investigated by injection of this peptide and measurement of its disappearance rate, or by observing the rate of its removal from fetal and maternal blood after delivery. Removal of hCG appears to involve two

components, the faster having a half-life of 5-15 hours and the slower half-life of 20-37 hours (Faiman et al, 1968; Yen et al, 1968; Rizkallah et al, 1969). Others (Parlow and Ward, 1961; Midgley and Jaffe, 1968; Wide et al, 1968) have recognized only one component. Geiger (1973) has tried to reconcile these differences by suggesting that the half-life of hCG changes sharply in the early puerperium. Clearance of hCG in the urine has been estimated variously from 0.4 to 6 ml/min., without any alteration in clearance rate related to the stage of pregnancy (Parlow et al, 1961; Rizkallah et al, 1969). This implies that changes in hCG levels in the blood during pregnancy must be due to alteration in secretion rate, not in clearance and degradation. About 5-25% of the circulating hormone appears in the urine.

Finally, there is evidence that hCG varies qualitatively during the course of pregnancy. Early in pregnancy, the carbohydrate content of the hormone and its biological activity are higher than in later pregnancy, although the immunological properties of the molecule remain unchanged (Vaitukaitis, 1976). Free alpha-subunits increase in concentration during pregnancy to about 300 ng/ml of serum whereas beta-subunits remain at about 50 ng/ml throughout pregnancy, which can be compared with concentrations of intact hCG of 6 µg/ml at its peak value (10 weeks) and about 1 µg/ml during the latter part of pregnancy. However, these concentrations may represent considerably greater synthesis of free alpha and beta-subunits, since the half life of the subunits after injection into female rats is

much shorter than that of intact hCG (Braunstein et al, 1972b).

vi) Physiological Role of hCG:

First, hCG plays an important role in steroid metabolism during pregnancy by stimulating the corpus luteum to secrete progesterone during the first 10 weeks and subsequently by promoting synthesis of the same hormone in the placenta(Gabbe et al,1971). Although in early pregnancy the pituitary no longer secretes luteinizing hormone (LH), apparently because of suppression of the appropriate hypothalamic releasing factor (LHRH) by hCG (Miyake et al, 1960), implantation of the embryo and early intrauterine embryo development are dependent on continued secretion of progesterone (Amoroso , 1955). The secretion into the maternal blood of large amounts of hCG in early pregnancy ensures a continuing stimulus to the corpora lutea to form progesterone until the fetoplacental unit takes over steroid production at a later stage in pregnancy. Direct evidence of such an action is shown by increased incorporation of ^{14}C-acetate into progesterone when slices of human corpora lutea are incubated with hCG (Savard et al, 1965).

After about the seventh week of pregnancy, the fetus and placenta (fetoplacental unit) play an increasing role in steroidogenesis. hCG appears to promote several stages of steroidogenesis in the fetoplacental unit. First, Villee has shown with incubated human placental preparations that hCG stimulated conversion of cholesterol to pregnenolone and progesterone (Villee et al, 1966; Gable et al, 1971). Second, synthesis of DHEA by the fetal adrenal appears to be stimulated

by hCG, as evidenced by increased excretion of DHEA in the urine of the newborn infant after administration of hCG (Lauritzen and Lehman, 1967; Lauritzen et al, 1969). This is likely to mimic the action of hCG during pregnancy, since there is a physiologically significant amount of hCG in the fetal circulation (Bruner, 1951), and net uptake of the hCG by the fetal adrenal has been demonstrated (Lauritzen et al, 1967).

There is evidence that hCG plays a role in the differentiation of the fetal gonads (Albert, 1969). Thus there is a close temporal relationship between the excretion of hCG and the morphological differentiation of the fetal testis (Kaplan et al, 1976) with accompanying hyperplasia of the cells of Leydig (Albert et al, 1953; Van Wagner et al, 1965) and a considerable elevation of plasma testosterone levels in male fetuses between 12 and 18 weeks of gestation (Abramovich et al, 1973). In confirmation of this, Abramovich et al (1974) have demonstrated that, during this period, hCG can stimulate androgen production by the testis in vitro. There may also be feedback control by testosterone of hCG programming, as evidenced by the lower hCG levels at term when the fetus is a male. Regarding effects of hCG on ovarian development, there is uncertainty about the alleged follicle-stimulating activity of hCG, which may be due to contaminating FSH of pituitary or placental origin (Jaffe et al, 1969; Parlow et al, 1970; Ashitaka et al, 1970). Thus a role for hCG in ovarian development remains unsubstantiated.

Finally, the alleged role of hCG as an immunosuppressive

agent during pregnancy is controversial. Several investigators (Kasakura, 1971; Keye and Jones, 1971; Contractor and Davies, 1973; Adcock et al, 1973; Teasdale et al, 1973) have demonstrated that hCG inhibited the stimulation of lymphocytes by phyto-hemagglutinin, in conformity with the observation that the lymphocytes of pregnant women are less responsive to this mitogen (Purtilo et al, 1972), and other evidence of impairment of immunological functions of hCG (Pearse and Kaiman, 1967; Nelson et al, 1967; Younger et al 1969). However, there are now a number of investigators (Gundert et al, 1975; Loke and Pepys, 1975; Pattillo et al, 1976) who interpret some of the above findings as being due to contamination of the hCG preparation with various immunosuppressants.

The mode of action of hCG at the cellular level has been explored mainly in relation to the ovary. In vivo studies (Lunenfeld et al, 1967; Espland et al, 1968) have shown that, following administration of iodinated hCG to animals, there is selective accumulation of the radioactive hormone in the ovary. In vitro studies with human ovaries demonstrated the presence of peptide hormone receptors in the plasma membranes of the ovarian cells (Lee and Ryan, 1973). Specific gonadotropin receptors with high affinity for hCG have been described in human corpora lutea and follicles, binding to which varies with the functional state of the ovary in relation to the menstrual cycle (Wardlaw et al, 1975). Such binding has been shown to result in formation of cyclic adenosine 3'5'-monophosphate (cAMP) through activation of adenyl cyclase

(Mason et al, 1973; Menon, 1974; Koch et al, 1974), in agreement with the general action of peptide hormones at the cellular level. The formation of cAMP is followed by activation of ovarian protein kinase (Vaitukaitis et al, 1975) and by phosphorylation of chromosmal proteins in the immature ovary (Jungmann et al, 1974).

In the case of the placenta, acceleration of steroid hydroxylation by hCG formed in the same cells has been attributed by Villee and Gabbe (1971) to an increase in NADPH availability caused by the hormone.

B. HUMAN PLACENTAL LACTOGEN

i) Discovery of Human Placental Lactogen:

At the time that "prolan" or hCG came to the attention of reproductive physiologists in 1930's, Madruzza in 1927 reported that when placental homografts were implanted into virgin guinea pigs, lactation ensued. These results suggested that the placenta contains substances other than gonadotropins. Subsequently, studies notably by Ehrhart (1936), Fukushima (1961), Kurosaki (1961), described the presence of a prolactin-like and a growth hormone-like substance in placental extracts. It was not until 1962, when Josimovich and MacLaren published their paper on human placental lactogen, also known as human chorionic sommato-mammotropin (hCS), that there was renewed interest in the placenta as an endocrine organ. The discovery of hPL by Josimovich et al was based on the finding that human

placental extracts partially cross-reacted with hGH antiserum in a double diffusion system. Using this immunological property, hPL has been purified to homogeneity by several investigators (Josimovich and MacLaren, 1966; Friesen, 1965; Florini et al, 1966; Catt et al, 1967; Li et al, 1971).

ii) Chemistry of hPL:

The hPL molecule is a single-chain polypeptide of 191 amino acids with valine and phenylalanine at the amino and carboxyl termini, respectively. The complete amino acid sequence, representing the work of three groups (Catt et al, 1967; Li et al, 1971, 1973; Niall, 1971, Niall et al, 1971; Sherwood et al, 1971) is shown in Figure 3. There are two intramolecular disulfide bonds linking half-cystine residues at positions 53 and 165, and 181 and 188, respectively. The protein contains no carbohydrate or lipid.

The molecular weight of monomeric hPL is 21,600 (Florini et al, 1966; Andrews, 1969; Li, 1970). Early studies on hPL indicated that it might occur as a dimer (Florini et al, 1966; Catt et al, 1967b), but other investigators suggested that dimers might be formed during extraction procedures (Andrews, 1969; Li et al, 1971). Hambley and Grant (1972) isolated both the monomeric and dimeric forms from pregnancy sera and from term and mid-gestation placental homogenates under a variety of conditions. About 3% of the hPL isolated has a molecular weight of 45,000, the remainder being 21,600. Furthermore, Schneider et al (1975a,b) were able to convert

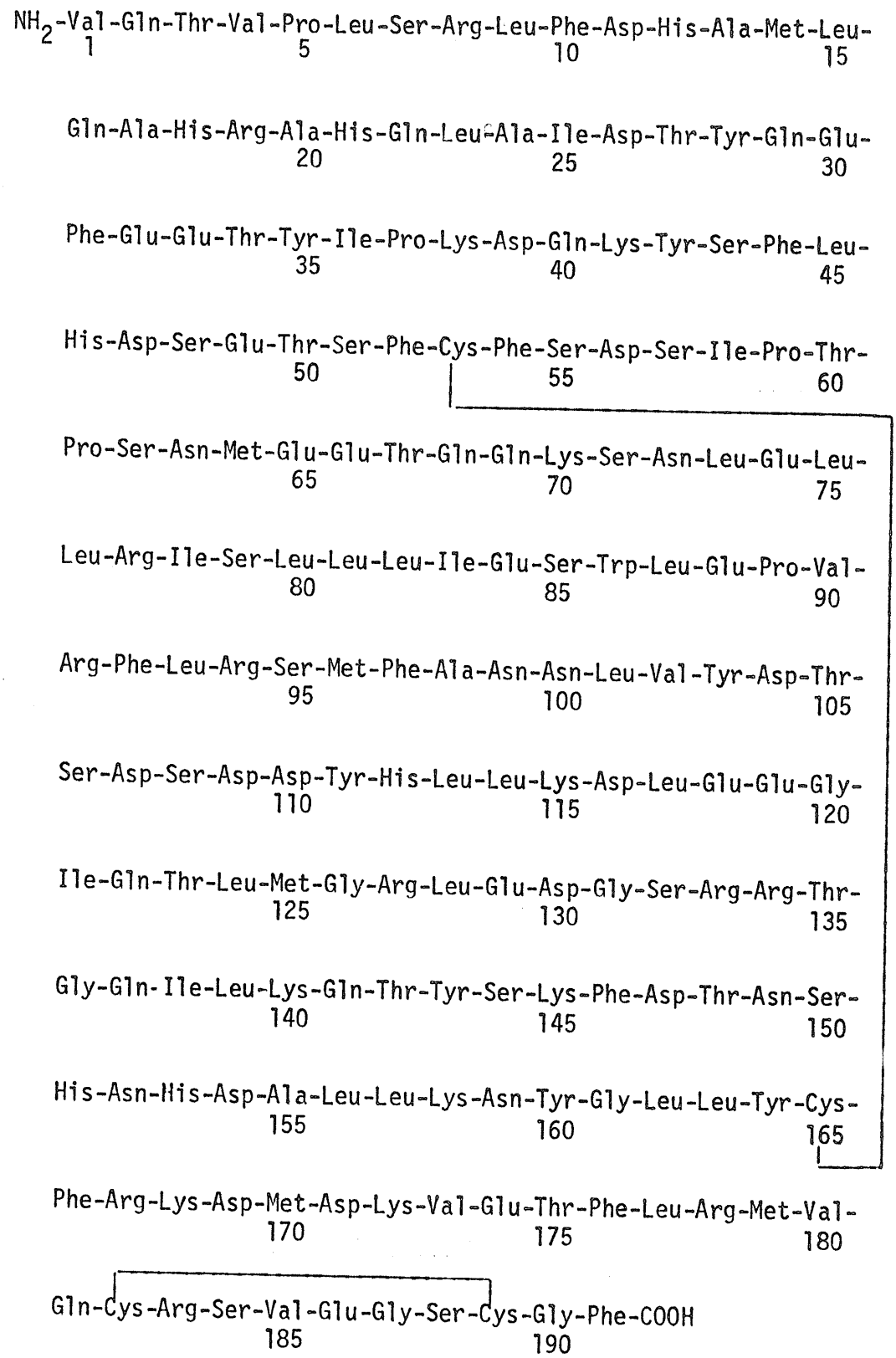

Figure 3. The amino acid sequence of the human placental lactogen molecule. The two lines indicate the two disulfide bridges. From the data of Li et al(1973)

the dimer by treatment with mercaptoethanol to a single protein band of molecular weight about 23,000. The large molecular weight form therefore appears to be a covalently linked disulfide dimer of native hPL chains. It appears that there is a larger precursor for hPL as described by Boime et al (1975, 1976) when placental mRNA was incubated in a wheat germ cell free, protein-synthesizing system. The evidence regarding this presursor is discussed in detail in the subtopic of "biosynthesis of hPL".

Heterogeneity among native hPL polypeptides has been postulated since the earlier isolation of hPL (Turtle et al, 1966; Neri et al, 1970). It was originally ascribed to deamidation of hPL molecules (Sherwood, 1967). However, Belleville et al (1972) identified a "slow" and a "fast-migrating" species on polyacrylamide gel electrophoresis and isoelectrofocusing of purified hPL. The forms were mutually interconvertible, the fast component being formed by reduction of SH groups with dithiothreitol, while the slow component contained oxidized disulfide bonds. Furthermore, Belleville et al (1973, 1976) were able to demonstrate that these forms are synthesized independently in vitro. They suggested that the fast-migrating molecule is thought to represent newly synthesized hPL, and the slow component is identified as stored hPL, which is only slowly released into the medium.

iii) Structure-Function Relationship:

Breuer (1969) originally reported that disruption of both the disulfide bonds of hPL abolished its

growth-promoting effect, whereas cleavage of only one bond did not lead to any loss of activity. Similarly, Neri et al (1972) observed a reduction of lactogenic activity in the pigeon crop-sac assay only when both disulfide bonds of hPL were cleaved. Aubert et al (1974) have observed a good correlation between molecular conformation of hPL and its biologic activity when they used derivatives that still contained the entire primary structure but had undergone some chemical modification; loss of tertiary structure was accompanied by loss of activity. However, these results conflict with those of another group (Aloj et al, 1972; Handwerger et al, 1972; Sherwood et al, 1972), who selectively disrupted the disulfide bonds, simultaneously destroying a methionine and a single tryptophan residue, and found that this treatment did not affect the lactogenic activity of hPL. However, its immunological potency was considerably diminished. These investigators concluded that, since the three-dimensional structure of hPL did not appear to be necessary for its lactogenic activity, this effect must reside in a small portion of the polypeptide chain. In support of this hypothesis, they found that cyanogen bromide treatment or performic acid digest of hPL retained lactogenic activity. Other studies in which hPL has been enzymatically cleaved also suggest that partial hydrolysis of the hormone does not destroy its biological activity. Neri et al (1972) demonstrated that tryptic digestion of four hPL bonds leads to only 30% loss of lactogenic activity and no reduction in somatotropic activity.

Deamidation, on the other hand, seemed to produce a small but statistically significant increase in the lactogenic effect of hPL (Handwerger et al, 1972; Handwerger et al, 1974).

The possibility of a relationship between the antigenic structure and biologic functions of hPL remains largely unexplored. Josimovich and Mintz (1968) proposed that the lactogenic and somatotropic properties of hPL may be associated with different antigenic sites on the molecule. Neri et al (1973) found that modification of the single tryptophan residue by either of two different methods resulted in complete loss of immunologic activity. However, one method abolished only the lactogenic activity of the hormone whereas the other affected both its biologic activities.

iv) Structural Relationship to hGH:

Friesen (1965) and Florini et al (1966) first noted the similarity in the amino acid compositions of hPL and human growth hormone (hGH). Both hPL and hGH are single chain polypeptides of 191 amino acid with two intra-chain disulfide bonds. A comparison of the complete amino acid sequence of hPL with that of hGH demonstrates some 85% homology (Sherwood, 1967; Sherwood et al, 1971, 1972; Li et al, 1971; Niall, 1971; Bewley et al, 1971, 1974; Handwerger and Sherwood, 1974).

The immunologic cross-reactivity between antisera to these hormones has been well documented. They exhibit a reaction of partial identity in the Ouchterlony immuno-

diffusion test. Josimovich and MacLaren (1962) suggested that hPL and hGH have two to three antigenic groups, respectively, only one of which is common to both proteins. This difference in antigenic sites is clearly shown by quantitative precipitin tests (Li, 1972). After modification of the tryptophan residue, hPL was unable to react with its homologous antiserum, but retained a limited capacity to bind anti-hGH, indicating that the tryptophan residue is not part of the antigenic determinants shared by the two hormones (Neri et al, 1973).

The primary structures of hPL and hGH are also very similar to that of ovine prolactin, which exhibits lactogenic but not somatotropic activity (Li et al, 1969, 1970; Bewley and Li, 1970, 1971). Niall et al (1971) have suggested that these biologically related hormones arose from a common ancestor, a shorter primordial peptide, by gene reduplication. Similarly, the structure of ovine growth hormone, which possesses growth-stimulating but not lactogenic activity, is partially homologous to that of hGH and hPL (Bewley and Li, 1974; Li et al, 1972).

v) Cell-origin of hPL:

Using both direct and indirect immunofluorescence techniques, Sciarra et al (1963) located hPL in the cytoplasm of the syncytiotrophoblast of chorionic villi, but failed to detect any specific immunofluorescence in the cytotrophoblast. This observation has been confirmed by others (Grumbach and Kaplan, 1964; Currie et al, 1966; Beck et al, 1969; Ikonikoff et al, 1971). Ikonikoff (1973) has demonstrated the ultra-structural location of hPL by means of a peroxidase-labeled

antibody. The peptide hormone was found on the ribosomes and in the cisternae of the rough endoplasmic reticulum and on the maternal surface of the plasma membrane of the syncytial microvilli. None was found on the Golgi apparatus or the mitochondria of the syncytium.

Thus, in the case of both peptide hormones, the syncytiotrophoblast appears to be the site of synthesis, in agreement with the structural features of this tissue, which are characteristic of a protein-secreting organ.

vi) Biosynthesis of hPL:

Studies on isolated placental preparations have established certain features of hPL production. Grumbach and Kaplan (1964) first demonstrated synthesis of hPL by fragments of first and third trimester placentas, and incorporation of labeled amino acid into hPL under these conditions has been observed (Gudson and Yen, 1967) even as early as day 29 of pregnancy (Gitlin and Biasucci, 1969). Friesen et al (1969) have concluded from studies on slices on human placenta with tritium-leucine that hPL is the major protein synthesized at term, and accounts for 5-10% of all proteins released from slices of human term placentas (Suwa et al, 1969a,b).

The properties of the lactogenic protein synthesized in vitro has been examined. Following incubation of human placental tissue, Friesen and coworkers (Suwa et al, 1969a,b; Friesen et al, 1972) found the predominant protein in the incubation medium to be identical to native hPL (M.W. 21,600),

whereas in the tissue extract, the principal component was a species larger than M.W. 100,000. However, no such species has been characterized in extracts from maternal serum obtained during pregnancy nor from placental tissue during hPL purification, and other workers (Neri et al, 1972; Genazzani et al, 1974; Rahman et al, 1974) reported that hPL synthesized by syncytiotrophoblast in tissue culture is identical to that isolated from placental tissue with a molecular weight of 21,600.

Synthesis of hPL by polyribosomes and mRNA isolated from placental tissue and incubated in vitro has been reported. Using a cell-free system containing placental ribosomes, Krebs II ascites tumor cell sap, and ^{35}S-methionine, Boime and Boguslawski (1974a) demonstrated in vitro synthesis of hPL, which was identical by tryptic fingerprints. The amount synthesized represented 5% of total protein made by early placental polyribosomes and 10% of that by full-term placental polysomes. However, in a subsequent study (Boime et al, 1974b), they used a specific radioimmunoassay to measure hPL and found that four times as much hPL was made by term placental ribosomes as by first-trimester incubations. Haustraete et al (1975) estimated by incubation with labeled amino acids and immunoprecipitation of the products that 7% of peptide synthesized on term-placental polysomes represent hPL. Chatterjee et al (1976) also examined the in vitro translation of early and full term placental polyribosomes and obtained no labelled hPL at

10 weeks of pregnancy, whereas 8% of all peptides made by term ribosomes were hPL. In conclusion, the various investigators cited above agree that between 7 and 10% of peptides made by term placentas is hPL, whereas little or none was identified in first trimester placental ribosomes. This pattern reflected the serum changes, in which hPL concentration increases as pregnancy progresses.

Placental mRNA has been extracted and translated in various systems. Samples from term placentas stimulated hPL synthesis much more extensively than does mRNA prepared from early placentas (Boime et al, 1976; Chatterjee et al, 1976), thus reflecting the relative abundance of hPL forming polyribosomes and eventually serum levels of hPL at these stages of pregnancy. Recently, McWilliams et al (1977) have used DNA complementary to the isolated hPL mRNA to quantitate specific mRNA sequences in the total RNA of early and late placentas, and confirmed the presence of 4 times greater abundance of hPL mRNA in the full term organ. This appears to be due to increased transcription, since the number of gene copies for hPL remains unchanged.

Since peptide hormones are often derived from precursor forms, such larger products of translation of hPL mRNA have been looked for. Using a wheat-germ translating system, Boime et al (1975, 1976) obtained a product of M.W. 25,000 and confirmed its identity by means of specific tryptic fingerprints. Cox et al (1976) also reported two hPL peptides formed by the wheat-germ system, but they

found products of molecular weights 21,000 and 19,000; the latter is thus smaller than authentic hPL (21,600), and it is consequently difficult to accept the former as the precursor molecule. Furthermore, Chatterjee et al (1976) were unable to identify the larger hormone molecule when placental mRNA was translated by the wheat-germ system. They also found by means of binding of ^{125}I-labelled anti-hPL that the polyribosome clusters with hPL peptide chains were compatible with a product of M.W. 21,000. However, Berken et al (1977) have recently identified a sequence of 19 a.a. at the N-terminus of the hPL peptide formed by translating hPL mRNA in a wheat-germ system. This sequence was similar in size and high leucine content to the signal peptide proposed by Blobel and Dobberstein (1975) as a common feature of the precursor or secreted proteins. Thus, the occurrence of a precursor form of hPL appeared to be demonstrable under some special conditions of translation.

vii) Regulation of secretion of hPL (in vitro)

In contrast to total protein released into the medium, which occurred at a constant rate, hPL release varied from one placenta to another (Suwa and Friesen, 1969b). This may account for the variable hPL levels in the serum of different pregnant women. Control mechanisms specific to hPL release may exist, as suggested by the observation (Gaspard and Franchimont, 1972) that placental slices incubated in vitro rapidly decrease in their ability to synthesize hPL while maintaining hCG secretion.

This is confirmed by Bolander et al (1978) that in placental explants, hPL synthesis remained constant while hCG synthesis increases with continued incubation. These studies suggested that a factor from the mother or fetus has been eliminated from placental explants which normally stimulates hPL secretion.

In contrast to hCG synthesis, addition of cAMP to term placental slices did not increase synthesis of hPL (Friesen et al, 1969; Handwerger et al, 1973; Belleville et al, 1974). Other potential regulators (insulin, progesterone, cortisol, and prostaglandin F_{2a}) were also found to be without effect by these authors. Prostaglandins E_2 and F_{2a} and the catecholamines have been found by other investigators to depress the synthesis and release of labelled protein by placental slices with a preferential effect on hPL (Belleville et al, 1974; Kraincanic et al, 1975). The inhibitory effect of prostaglandin F_{2a} observed by the Kraincanic's group can be correlated with the reduction in plasma hPL observed in patients undergoing therapeutic abortion with this agent (Speroff et al, 1972; Ylikorkala and Pennanen, 1973). It is interesting that catecholamines (dopamine) have the ability to suppress the secretion of hPL in vitro, whereas dopamine-antagonist elevated the secretion of hPL. These studies suggest that the secretion of hPL might be similar to the secretion of prolactin from the pituitary under the control of catecholamines. However, the above findings have not been substantiated.

viii) Secretion and metabolism of hPL:

hPL is detectable in maternal serum from about 6 weeks of gestation until term. The concentration rises linearly to a plateau at 34 weeks and at term is about 5-10 µg/ml of serum. In the placenta, hPL appears somewhat earlier, being detectable by immunofluorescence in the trophoblast at about 12 days (Beck, 1970). In placental tissue, the concentration of hPL varies at 20-100 mg/kg wet weight at various stages of pregnancy (Josimovich, 1968a). From studies of turnover of hPL, it has been estimated that the placenta produces 0.3-1 g of hPL per day during the third trimester (Beck and Daughaday, 1967; Kaplan et al, 1968).

hPL is essentially absent from urine (Rochman et al (1972). Rochman et al found that the disappearance rate of hPL decreases in nephrectomized dogs. Other sites of degradation are unknown, but the elevated levels of hPL in cases of liver disease in pregnancy (Singer et al, 1970; Ylikorkala, 1973) suggested that the liver is involved in removal of the hormone. Further evidence suggesting that the liver might be involved in degradation of hPL is derived from the report that human liver slices could specifically bind hPL (Carr and Friesen, 1976). The disappearance of hPL from the blood has been resolved into two components, one with a half-life of 10-15 minutes and a second one of 30-60 minutes (Kaplan et al, 1968; Beck et al, 1974).

The mechanisms regulating hPL secretion remain obscure. Josimovich (1968a) considered the increase in serum hPL levels to be directly correlated with the mass of placental tissue, and MacMillan et al (1976) support this by finding that mothers with small infants have reduced plasma levels of hPL correlated with the small placentas. This view is supported by the findings of Spellacy et al (1971a) that cases of toxemia of pregnancy frequently have low levels of hPL associated with small placentas, and Tyson et al (1974) described two diabetics with elevated hPL levels and larger placentas than six other controlled diabetics who had normal hPL levels. High levels of hPL are recorded in twin pregnancies (Ylikorkala, 1973) and Tyson et al (1974) have claimed that the degree of elevation can be correlated with the size of the placental mass of the twin fetuses. Other studies (Sciarra et al, 1968; Saxena et al, 1969; Singer et al, 1970) failed to show a correlation between placental weight and hPL level either at term or earlier in pregnancy. The data of two other groups (Genazzani et al, 1972; Rolschau et al, 1975) may resolve some of this discrepancy by suggesting a correlation between placental weight and hPL between 38 and 40 weeks of gestation, but not before this time. Thus in early pregnancy other factors may regulate the secretion of hPL.

Attempts have been made to relate plasma levels of hPL to various maternal factors such as age, parity, physical activity, and food ingestion with negative results. Some

metabolic factors may influence hPL output by the placenta. Although some investigators (Spellacy et al, 1966; Grumbach et al, 1968; Fairweather, 1971; Ajabor and Yen, 1972; Tyson et al, 1974) found no change in hPL levels due to glucose administration to pregnant women, others (Burt et al, 1970; Spellacy et al, 1971a; Gaspard et al, 1973, 1974) observed a significant fall. Conversely, hypoglycemia has been found to elevate hPL levels (Kim and Felig, 1971; Spellacy, 1971; Gaspard et al, 1974; Tyson et al, 1971a,b, 1974). On the other hand, hPL levels in plasma appear to be unaffected by the free fatty acid concentrations in the blood (Morris et al, 1974; Gaspard et al, 1975) or by PGF_{e2} and PGF_{2a} (Keller et al, 1972), although the latter may affect hCG output.

ix) Biological effects and physiological roles of hPL:

This hormone appears to have three major effects during pregnancy, all directed toward maternal metabolism. First, it is luteotropic, as evidenced by its stimulation of progesterone and estrogen secretion by the corpus luteum of hypophysectomized pseudopregnant rats, an effect that is potentiated by administration of hPL and hCG together (Josimovich, 1968b). Thus, hPL secretion during the second trimester of human pregnancy may enhance the action of hCG on the corpus luteum. However, attempts to demonstrate such an action in pregnant women have not been successful (Stock et al, 1971).

Second, placental lactogen stimulates milk

production (casein synthesis) by rabbit mammary gland (Josimovich and MacLaren, 1962; Josimovich and Brande, 1964; Friesen, 1966; Turkington, 1971) and simian mammary gland (Beck, 1972). It also stimulates the pigeon crop-sac analogous to the action of pituitary prolactin (Josimovich and MacLaren, 1962; Forsyth, 1970; Li, 1972). The lactogenic potency of hPL had been assessed by various assays. In pigeon crop-sac assay, hPL has 10% the potency of sheep prolactin (Forsyth, 1970; Li, 1972), but 50-100% of the activity of sheep prolactin in stimulating casein synthesis in rabbit and mouse mammary gland explants (Forsyth, 1971; Turkington, 1971; Kleinberg et al, 1971). hPL and prolactin compete for the same receptors in rabbit mammary gland cell membranes, and hPL is 30-50% as potent as hPRL (Shiu et al, 1973), but their relative roles in mammary development are still unclear. Since hPL is present at term in concentrations 35 times greater than those of hPRL (Hwang et al, 1971; Friesen, 1971), it is possible that hPL may be more important. Leader (1975) has postulated that the primary action of hPL is to stimulate the development of the mammary gland during pregnancy, without causing milk secretion due to its blockade of prolactin receptors; at birth, withdrawal of hPL (hence withdrawal of the blocking effect on prolactin receptors) then results in milk secretion under the active stimulus of prolactin of pituitary origin. Although Leader's hypothesis is interesting, unfortunately there is no report of lactogenic receptors in human mammary gland.

Although hPL simulates the action of pituitary growth hormone, it is being independent of the homeostatic mechanisms that regulate hGH production. It is secreted during later pregnancy in amounts two orders of magnitude greater than that of hGH, and at a relatively constant rate throughout the day. In various tests in animals (Josimovich and MacLaren, 1962; Florini et al, 1966; Josimovich, 1966; Friesen, 1965; Li, 1972), hPL showed a distinct but smaller growth promoting activity than hGH, approximately 3-13% of the activity of hGH. In man, the growth-promoting activity of hPL is controversial. Evidence of its effect on pituitary dwarfs (Grumbach and Kaplan, 1964; Grumbach et al, 1966, 1968; Schulz and Blizzard, 1966; Josimovich, 1968b, Schuttaine and Drash, 1972) and in normal subjects (McGarry and Beck, 1972), either given alone or in combination with hGH, was inconclusive, but it is generally agreed that growth promoting potency of hPL in man is minimal.

The possible role of hPL in human is not clear, however, Grumbach and coworkers (1968, 1974) suggested that a major function of hPL is to so alter maternal metabolism that adequate supplies of glucose, amino acids, and minerals are made available to the fetus during the latter part of pregnancy, when fetal requirements are rapidly increasing. In the mother, there are no gross changes in carbohydrate or lipid metabolism during the first trimester, but in the last trimester there is diminished responsiveness to insulin, impaired glucose tolerance and mobilization of

lipid stores (Picard et al, 1968; Fairweather, 1971). Prolonged infusion of hPL into normal or hypopituitary subjects resulted in increased free fatty acid levels and impaired insulin level following glucose administration (Beck and Daughaday, 1967; Samaan et al, 1968). However, the changes in blood sugar response are disputed by Josimovich and Mintz (1968), and the changes in free fatty acid levels were not observed by Kalkhoff et al (1969). Studies on animals treated with hPL and in vitro experiments confirm the increased secretion of insulin (Martin and Friesen, 1969; Lopez et al, 1971) and the release of free fatty acids from adipose tissue (Turtle and Kipnis, 1967; Genazzini et al, 1969; Felber et al, 1972; Mochizuki et al, 1975). The benefits to the fetus of this mobilization of substrates are illustrated by experiments in which hPL was given to pregnant rats resulting in increased fetal weight, glycogen, protein, and lipid content (Mochizuki et al, 1973). No such response was observed when hPL was administered directly to the fetus. Finally, there is some evidence (Spellacy et al, 1971a; Kim and Felig, 1971; Laube et al, 1972; Gaspard et al, 1975) that the level of maternal blood sugar provides a negative feedback system for controlling hPL secretion rate.

C. OTHER HUMAN PLACENTAL PEPTIDE HORMONES AND SECRETED PROTEINS.

(i) Human Chorionic Thyrotropin (hCT):

A thyrotropic substance was first isolated from term human placentas by Hennen in 1965. Like pituitary TSH, hCT stimulates thyroid hormone secretion when injected into mice and increases uptake of inorganic phosphate into phosphorylated compounds. hCT has been found immunologically to be much more closely related to bovine and porcine TSH than to human TSH, so that its biological activity is more effectively neutralized by antibovine TSH than by antihuman TSH (Hennen et al, 1974; Hennen, 1975; Hennen and Pierce, 1969). hCT is a glycoprotein and has a molecular weight of 28,000 as determined by gel filtration (Hershman and Staines, 1971). However, unlike TSH, which has an alpha-chain identical in peptide sequence to that of hCG, hCT probably does not contain the same alpha-chain, as demonstrated by the failure to cross-react with hCG or hCG-alpha subunits in homologous radioimmunoassay systems (Nistula et al, 1973; Tojo, 1975). Analysis of purified hCT by Tojo et al (1973) indicates an overall amino acid composition quite distinct from the hCG, and a much lower sugar content (3.5% versus 30%). The primary sequence of hCT has not been determined.

Unlike the plasma concentrations of hTSH which remained within non-pregnant limits, hCT became detectable early in pregnancy with an average level of 7 μg/ml plasma

and rose to about 30 μg/ml by the seventh month, where it remained for the rest of pregnancy (Tojo et al, 1973; Kanazawa et al, 1976). In cases of hydatidiform mole with elevated hCT levels delivery was followed by rapid reduction in plasma hCT level ($T_{\frac{1}{2}}$ of the order of 12 hours), in contrast to the much slower fall in plasma levels of hCG in the same patients. Injection of hypothalamic thyrotropin-releasing hormone (TRH) has been found to raise the plasma level of hTSH but not of hCG (Kanazawa et al, 1976; Hershman and Burrow, 1976). It is not clear at present what controls the secretion of hCT.

The role of hCT during gestation has yet to be defined. While pregnancy is not usually characterized by hyperthroidism, thyroid function is known to be somewhat altered. The thyroid is frequently enlarged (Freedberg et al, 1957; Myant, 1964). Serum thyroxine-binding globulin increased, due to high levels of circulating estrogens, resulting in elevated serum protein-bound iodine (Peters et al, 1948; Dowling et al, 1956). However, the secretion rate and the concentration of free thyroxine remain the same (Oppenheimer et al, 1963; Ingbar et al, 1965; Dowling et al, 1967). A possible role for hCT in the development of the fetal thyroid is suggested by the finding (Shepard, 1963, 1968) that the fetal thyroid is active by 10 weeks of gestation, prior to the production of TSH by the fetal pituitary.

Hyperthyroidism, resulting from increased thyroid

stimulating activity, is found in patients with molar pregnancies and choriocarcinomas (Dowling, 1960; Odell et al, 1963; Koch et al, 1966; Mann et al, 1967; Cohen et al, 1970; Galton et al, 1971; Tojo et al, 1973), as well as other nonchorionic cancers (Hennen, 1966a, b, 1974) and is unresponsive to TRH (Miyai et al, 1966). The thyrotropin extracted from tumor tissue (Hennen, 1966a, b, 1967; Hershman et al, 1970) differs from hCT in molecular size and immunological properties. Recent evidence (Nisula and Ketekselger, 1974; Kenimer et al, 1975) suggests that a major substance in hydatidiform moles is chorionic gonadotropin. However, this may not be an adequate explanation of the thyrotropic action of these trophoblastic tumors, since the levels of hCT and hCG in plasma decline at quite different rates following removal of the trophoblastic disease (Tojo et al, 1973). In addition, although Nisula et al (1974) reported that purified hCG has thyroid-stimulating activity, Saida et al (1977) has shown that, whereas their hCT preparation stimulates cAMP formation in slices of guinea pig thyroid gland, purifed hCG does not. Nevertheless, the question of elevated thyrotropic activity during pregnancy is due to an intrinsic activity of hCG(and hLH) or due to hCT remains unclear.

(ii) Human Chorionic Follicle-Stimulating Hormone (hCFSH):

In addition to luteotropic activity due to hCG, placental extracts also contain follicle-stimulating activity (hCFSH) (Ashitake et al, 1970, 1972; Tojo et al, 1975). Although even highly purified hCG still retains weak FSH activity (Louvet et al, 1976), persuasive evidence is provided by Tojo and his colleages that there is a separate hCFSH peptide which contaminates crude hCG preparations. Maximium activity of hCFSH was found in first-trimester placentas. The purified peptide hormone contains 32% sugar like hCG, but differs from hCG in chemical structure and immunological properties and cross-reacts with antisera to pituitary hFSH (Ashitake et al, 1972). Like FSH of pituitary origin, but unlike hCG, it stimulates cell division in the ovary (Tojo et al, 1975). Culture of explants from early placenta with ^{3}H-proline and ^{14}C-glutamic acid demonstrated independent synthesis of hCFSH as well as hCG (Maruo, 1976).

(iii) Human Chorionic Corticotropin (hCC):

Evidence regarding adrenocortical-stimulating activity from placental sources have been published over the past 24 years. As summarized by Genazzani et al (1975), several authors have observed increased levels of free cortisol and ACTH in the plasma of pregnant women, but others failed to confirm this. The occurrence of adrenocorticotropic activity in placental extracts has also been reported by a number of authors, but in reviewing these, Saxena (1971) could not exclude ACTH present in maternal blood contaminating the placental extracts. However, recently Genazzani et al (1974, 1975) have demonstrated accumulation of ACTH immuno-reactive components in slices of placental, and Rees et al (1975) observed an increase in plasma levels of ACTH during pregnancy that could not be suppressed by administration of dexamethasone, which is well known to inhibit ACTH release from the pituitary gland. According to Genzazzani et al (1975) and Rees et al (1975), use of optimal new techniques for measuring plasma ACTH shows progressive increases in ACTH throughout pregnancy to near term with maximal levels three to four times above normal (60 pg/ml) compared to 16-20 pg/ml in nonpregnant women). The ACTH content of placental extracts reported by Genazzani et al (1975) corresponds to about 7 μg per placenta, which is much lower than the amounts of hCG and hPL recovered from placentas.

(iv) Other Secreted Placental Proteins:

There are reports of other peptide hormones made in the human placenta. A number of authors have described melanocyte-stimulating activity, and others report the presence of oxytocin, vasopressin, relaxin and renin. None of these has reached the point of purification and rigorous identification. A new hormonal activity, uterotropic placental hormone (UTPH) has been reported (Beas et al, 1975) in which a partially purified placental protein has been found to cause growth of mouse uteri . This protein has been detected in the blood of pregnant, but not of nonpregnant, women.

A number of plasma proteins of no known function have also been identfied in serum of pregnant women and have been especially studied by Bohn (1974a, b) and by Lin et al (1974, 1976a, b). The latter correlate their nomenclature for these pregnancy-associated proteins (PAPPs) with some of the pregnancy proteins of Bohn and other investigators : PAPP-A, PAPP-B, and PAPP-C (similar to Bohn's SP_1) appear to be synthesized by the placenta, since they are absent from the plasma of nonpregnant women and increase in amount during the second half of gestation, though at different rates (Lin et al, 1974). The PAPPs also disappear from the plasma after delivery, with half-life varying from 1-4 days. Finally, fluorescent antibodies to PAPP-A and PAPP-C reveal that these proteins are present in abundance in term placentas, notably in the

syncytiotrophoblast (Tatarinov et al, 1976; Kin and Halbert, 1976). In contrast, a number of other plasma proteins increase during pregnancy, but are not absent from plasma of non-pregnant subjects. One such is the "pregnancy-zone protein" (PZP, similar to SP_3 of Bohn), which is found in placenta in proportion to the amount of retained maternal blood; furthermore, immunofluorescent staining for this protein has localized it to the blood vessel walls in the placenta (Lin and Halbert, 1976).

Finally, the placenta is the site of formation of the heat stable alkaline phosphatase found in the blood of pregnant women in the latter part of pregnancy (Fishman and Ghosh, 1967). It has a molecular weight of 58,000 including 30% sugar residues (Sussman and Gottlieb, 1969) and appears on the plasma membrane covering the microvilli of the placental surface, from which it is continuously released into the maternal circulation as the membrane undergoes renewal (Hubstaert et al, 1973). In the plasma, the enzyme has a slow turnover rate (Clubb et al, 1975), so that the plasma level is maintained although the placenta contains relatively small amounts of the enzyme (Harkness, 1968). With regard to the significance of the changes in plasma levels of this enzyme, Oesterling et al (1977) found that the level was elevated in mothers receiving insufficient dietary energy and that this elevation was moderately correlated with a reduction in birth weight of the infant. They therefore postulate that an elevation in maternal cortisol levels due

to malnutrition induces higher levels of enzyme in the placenta and this was reflected in more extensive release into the circulation.

Ib SUBPRIMATE PLACENTAL PROTEIN HORMONES

Introduction:

In this review, an attempt has been made to draw attention to the placental protein hormones (chorionic gonadotropin and placental lactogen) of one subprimate species, the rhesus monkey (Macaca mulatta), since the information on the excretion of these two placental protein hormones in this species is quite extensive compared with that from other sub-primates.

A. Monkey Chorionic Gonadotropin (mCG)

(i) Discovery and Secretion Pattern of mCG:

In 1928, when Ascheim and Zondek reported that their test for pregnancy in women was positive, they found that it was also positive with the urine of the pregnant rhesus monkey. Later Ehrhardt and Ruhl (1933) confirmed Ascheim and Zondek's report using samples from pregnant rhesus monkeys. Urine samples from seven pregnant monkeys were also tested and the AZ reactions were positive in each case. No details were given about the time of pregnancy. Subsequently, various investigators (Hamlett, 1937; Defts, 1941; Simpson, 1955) using various bioassays confirmed that monkey chorionic gonadotropin was present in urine and blood samples at about 20 to 34 days but was not present before or after these days.

Tullner and associates (Tullner et al, 1966a, b;

Tullner, 1968) were the first to make semi-quantitative estimates of the concentration of CG in the serum and urine of the intact and ovariectomized rhesus monkey in early pregnancy by mouse uterus bioassay using hCG as standard. When two hundred and fifty-seven serum samples from 136 pregnant monkeys were collected from the 9th to the 48th day after mating and assayed for CG activity, CG was first found in the serum 15 days after mating, and was present in all sera until the 28th day after mating. With one exception, mCG was not found in sera collected 35 days after mating. Maximum amounts of serum gonadotropin, equivalent to 3.4 I.U. hCG per ml, were found between the 21st and 30th days after mating. When urinary mCG was determined on 24-hour collections from five pregnant monkeys between the 21st and the 41st day after mating, the greatest amounts of mCG were excreted also between 21st and 26th days of pregnancy. Similar results were also obtained by Arslan et al (1967).

With the discovery that the chorionic gonadotropins from monkey will react in immunoassay systems which were designed to measure gonadotropins of human origin (Tullner et al, 1969; Nixon et al, 1971; Wide et al, 1971), monkey chorionic gonadotropin in the serum, urine and placental extracts has been quantitated. By employing the radio-immunoassay, Hodgen et al (1974) measured mCG in the serum and urine from 8 to 12 days after the conception. When serum progesterone was measured simultaneously in these

samples, significant elevations were noted 24 to 48 hours after the appearance of mCG in serum. These findings suggested that the attaching blastocyst or the developing syncytiotrophoblast possesses or rapidly attains the capacity to secrete mCG. The presence of mCG at the time of implantation probably maintains the viability of the corpus luteum and temporarily enhances progesterone secretion immediately following implantation. Similarly, using this RIA, Hodgen et al (1975) also quantitated the chorionic gonadotropin production by the placenta of the rhesus monkey. They found that mCG was detected in extracts of rhesus monkey placentas as early as 22 days after fertilization, but was not detectable in the placenta after 40 days of gestation.

More recently, Walsh et al (1977) again measured plasma mCG from pregnant monkeys at 22, 42 and 157 days of gestation using ovine LH as standard. They found that the levels of mCG at day 22 of pregnancy were approximately 250 ng/ml; however, during the latter stages of gestation mCG were either nondetectable or less than 0.7 ng/ml. Thus, their findings confirm the results of Hodgen et al (1974).

No information exists on the factors which regulate the synthesis and secretion of mCG.

(ii) Chemistry and Cell-origin of mCG:

At the present, the amino acid composition and primary amino acid sequence of mCG are not known.

Using immunofluorescent staining techniques, the mCG was localized in the syncytiotrophoblast (Pierce et al, 1964; Luckett, 1970).

(iii) Immunological Properties of mCG:

Antibodies generated against hCG in rabbits have been used to examine antigenic similarities among primate chorionic gonadotropins from serum or urine (Tuller et al, 1969; Nixon et al, 1971). Recent studies by Chen et al (1976) demonstrated that when chorionic gonadotropins from human (hCG), chimpanzee (chCG), gorilla (gCG), organgutan (orCG), baboon (bCG), macaque (mCG) and marmoset (maCG) were investigated in a radioimmunoassay system using an antiserum with antigenic determinants known to reside in the terminal 15 amino acid residues of the unique carboxyl-terminal peptide of hCG-beta without cross-reactivity to hLH, there is close antigenic similarity among hCG, chCG and gCG but minimal similarity with other simian CG's.

(iv) Physiological Role of mCG During Pregnancy:

In a series of experiments in which hCG was administered into the rhesus monkeys during the luteal phase, the length of the menstrual cycle was significantly prolonged (Hisaw, 1944). When he compared the period

required for the chorionic gonadotropin production and the development of the chorionic villi in the rhesus monkey, a close correlation was found. Since Hartman (1941) had presented evidence that the corpus luteum of pregnancy was no longer required after gestation day 25, Hisaw speculated that chorionic gonadotropin might act by assuming the luteotrophic function of the pituitary at a time when the corpus luteum was involuting and menstruation was imminent. Thus, chorionic gonadotropin would maintain luteal function until the placenta could carry on the hormonal function of the corpus luteum.

A marked rise in plasma progesterone occurs in rhesus monkeys about the time of implantation (Neill et al, 1969). This rise precedes the earliest detectable levels of mCG in blood or urine with the most sensitive bioassay methods (Arslan et al, 1967). However, in a recent study employing a radioimmunoassay method for the rhesus monkey chorionic gonadotropin, progesterone and mCG levels increased concomitantly (Atkinson et al, 1971; Hodgen et al, 1974; Walsh et al, 1977). In fact, when mCG levels have attained a peak at conception day 20-22, progesterone levels have reached a nadir (Tullner, 1968; Hodgen et al, 1974). Furthermore, Neill and Knobil (1969) reported that when hCG (25 IU per day) was administered to rhesus monkeys in the luteal phase of the menstrual cycle a rise in plasma progesterone levels occurs but with continued treatment, the corpus luteum becomes refractory as indicated by a

decline in progesterone levels. These studies demonstrated a close association between mCG and progesterone production from the corpus luteum.

A functional corpus luteum of pregnancy is indispensable before conception day 21 in rhesus monkeys. Ovariectomy before this time is followed by abortion (Tullner and Hertz, 1966). Meyer et al (1969) have shown that progesterone treated monkeys, ovariectomized between the second and sixth days after ovulation, were able to provide the necessary milieu for implantation and maintenance of gestation. Urinary mCG was produced in all but one of the monkeys. Nevertheless, progesterone in the absence of the pituitary cannot support early gestation. When female monkeys were hypophysectomized between the 19th and 22nd day of gestation, pregnancy was not maintained. Progesterone treatment of similar experimental animals extended pregnancy for about one month (Arslan et al, 1969). Hypophysectomy does not terminate pregnancy when performed at 5 weeks in rhesus monkeys (Smith, 1954). These experimental results emphasize the critical role of ovarian progesterone in maintenacne of early pregnancy and the association between the beginning of chorionic gonadotropin synthesis and the maintenance of the corpus luteum. Therefore mCG is an essential luteotropic hormone during early pregnancy.

B. MONKEY PLACENTAL LACTOGEN (CHORIONIC SOMATOMAMMOTROPIN)

(i) Introduction:

Agate (1952) and Smith (1954) were the first to suggest that monkey placentas might secrete mammotropic substances. Their suggestions were based on the observations that when rhesus monkeys were hypophysectomized during pregnancy (mid-gestational period), hypophysectomy did not cause abortion, and mammary glands developed normally. Furthermore, transient lactation occurred at parturition.

In 1964 Kaplan and Grumbach reported the monkey placenta contains a substance that cross-reacts with antisera with hGH and hPL, and subsequently this substance has been isolated and purified to homogeneity by several investigators (Grant et al, 1970; Shome et al, 1971; Vinik et al, 1973). Because this substance has both mammotropic and somatotropic activities as demonstrated in experimental animals, it has been named chorionic somatomammotropin or placental lactogen.

(ii) Chemistry and Immunological Properties of mPL:

Whether mPL consists of one component or two components is still debatable. Shome and Friesen (1971) reported that there are two components, mPL-I and mPL-II, whereas Grant et al (1970) and Vinik et al (1973) reported that there is only one component. Nevertheless, these groups all agreed that the molecular weight of mPL is approximately 21,000 to 22,500. Amino acid composition of mPL is similar to

that of hPL and hGH (Shome and Friesen, 1971). The primary structure of mPL has not yet been determined.

Immunochemical studies of mPL, hPL, hGH, reveal that mPL cross-react to a greater extent with antiserum to hGH than does hPL (Belanger et al, 1971; Vinik et al, 1973). In the radioimmunoassay for mPL, placental extracts from all monkey species cross-reacted equally well, whereas human and monkey growth hormone, and hPL reacted poorly, and pituitary prolactins do not cross-react at all in the assay system (Friesen et al, 1971; Vinik et al, 1973).

(iii) Cell-origin and Pattern of Secretion of mPL:

Friesen (1968) is the first one who studied the biosynthesis of mPL from monkey placentas. Using immunofluorescent staining technique (antibodies to hGH), it has been demonstrated that mPL is synthesized in the cytoplasm of the syncytiotrophoblast (Currie et al, 1966; Ikonicoff et al, 1971).

Using radioimmunoassays for mPL, it was found that the secretion of mPL and hPL are very similar. mPL was detectable at 22 days of gestation, however, at day 42 the mPL titers averaged 1.5 µg/ml and increased throughout pregnancy to reach a mean concentration of 11 µg/ml at term, whereas mPL in umbilical vein blood was less than 100 ng/ml (Belanger et al, 1971; Walsh et al, 1977). After delivery, maternal mPL concentrations decreased rapidly with a half-time disappearance rate of 20 minutes as compared

with 13 minutes for hPL. The second half-life of mPL is 36 hours as compared with 40 minutes for hPL (Friesen et al, 1971). The estimated production rate of mPL is 0.36 g/day, which is in contrast to 1.09 g/day reported for hPL. The placental concentration of mPL (μg/g) wet weight) is 10% that of hPL (Kaplan and Grumbach, 1974).

Experiments of foetoectomy performed by Friesen et al (1971) demonstrated that the foetus did not exert any acute control over mPL production, but may influence the rate of placental growth and indirectly the production of mPL. Like hPL, there is virtually no information on the factors which regulate the synthesis and secretion of mPL.

(iv) Biological and Physiological Roles of mPL:

There is no information on the roles played by mPL in the monkey during pregnancy. In limited studies on the somatotropic activity of mPL (Shome and Friesen, 1971) demonstrated that mPL appears to have a greater somatotropic activity than hPL. Others (Josimovichet al, 1964) found that mPL has a biologic potency of the same order of magnitude as hPL in the mouse vaginal mucification luteotropic assay.

II NON-PRIMATE PLACENTAL PROTEIN HORMONES

IIa NON-PRIMATE PLACENTAL GONADOTROPINS

Introduction:

It is still not certain whether placental gonadotropin is secreted by the placenta of non-primates with the exception of pregnant mare serum gonadotropin.Although numerous data and experimental evidence suggest that gonadotropin might be secreted by the placenta of the rat(Pencharz et al,1933; Selye et al,1933; Astwood et al,1938; Averill et al,1950; Matthies,1965,1966,1967,and 1971; Cheng,1975; Haour et al,1976), mouse (Newton et al,1939; Deansely et al,1941), cat (Asdell,1965; Sadley,1975), dog(Holst et al,1971; Smith et al,1974), goat (Van Rensberg,1971), and the sheep (Denamur et al,1973; Moore and Rowson,1966a,b,c,d; Amoroso,1952; Bjorkman,1965; Davies et al, 1966; Boshier,1969), none of these putative chorionic gonadotropins has ever been purified for chemical analysis. Since pregnant mare serum gonadotropin(PMSG) and rat chorionic gonadotropin are the ones which have been most intensively described, in the following section , I wish to confine discussion to these two placental hormones only, whereas other putative chorionic gonadotropins in other species will be discussed in conjunction with placental lactogens.

Pregnant Mare Serum Gonadotropin (PMSG)

Before the concept of a "placental gonadotropin" was well established, Cole and Hart (1930) annouced their finding of gonadotropic activity in the serum of the pregnant mare. Zondek(1934) detected this activity almost simultaneously. As in the case of hCG, an extensive literature rapidly developed around this factor which soon became known as pregnant mare serum gonadotropin (PMSG). Later studies showed that PMSG is similar to hCG but with a somewhat different ratio of LH to FSH-activity. An origin in maternal tissue was claimed for PMSG by Clegg et al (1934) and by Gonzalez-Angulo et al (1972). A recent report by Allen et al (1972) presented evidence for chorionic origin which consisted of in vitro production of the hormone by chorionic girdle cells.

The molecular weight of PMSG remains uncertain. Bourrillon and Got(1959) obtained a molecular weight of 28,000 for PMSG employing sedimentation analysis. However, size determination by means of polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS) suggests PMSG may have a molecular weight as high as 53,000 (Gospodarowicz, 1972). Papkoff(1974) recently reported the dissociation of PMSG into alpha and beta subunits which possessed very low biological activity. Recombination of these subunits restored both the FSH-like and LH-like biological properties. Bourrillon and Got (1957) employed zone electrophoresis to obtain an isoelectric point of 1.8 for PMSG. The carbohydrate content of PMSG is higher than that of the other gonadotropins, comprising nearly 50% of the weight of the molecule. The sialic acid content of PMSG is high(Bourrillon et al,1959; Gospodarowicz,1972) and small quantities of glucose have been reported (Jutisz,1972).

RAT CHORIONIC GONADOTROPIN:

Until now, there is no compelling evidence for the existence of rat chorionic gonadotropin(rCG). Although early studies by Pencharz and Lyons(1933), Selye et al(1933), Astwood and Greep (1938), and Averill et al(1950) demonstrated that rat placentas possess luteotropic property with the highest activity in day 12 placentas, subsequent studies by Matthies (1965,1966,1967,1971), Cohen et al(1969) demonstrated that the mammotropic activity is also present in the day 12 placental extracts and maternal sera . Thus these investigators suggest that the luteotropic activity of the placentas detected by early studies may be due to rat chorionic mammotropin or rat placental lactogen, since pituitary prolactin is also shown to be luteotropic. The most convincing piece of evidence for the existence of rat chorionic gonadotropin is provided by Cheng(1975) and Haour et al(1976). By employing radioreceptor assay for gonadotropin(RRA-LH), Cheng(1975) identified rat chorionic gonadotropin from day 12 placental extracts but not on other days of pregnancy. The elution volume of rCG after gel filtration on Sephadex G-100 was identical to the larger species of rat chorionic mammotropin and ^{125}I-rat luteinizing hormone (30,000 to 32,000 M.W.). Immunologically, rCG cross-reacted poorly with rabbit anti-rLH but the slope of the dilution-curves of both rCG and rLH was the same. However, rCG has not been purified and its chemical properties remain unclear.

II b. NON - PRIMATE PLACENTAL LACTOGENS

Introduction:

There are various experimental manipulations which may provide evidence for the existence of placental lactogen in a given species. Therefore, I wish to discuss briefly the methods used to study placental lactogen. Emphasis will be on radioreceptorassays, since several non-primate placental lactogens have been successfully purified by employing these techniques to monitor the hormonal activities.

(i) Hypophysectomy in Pregnancy:

If pregnancy continues after hypophysectomy, maintenance of mammary development and the occurrence of a transient lactation at parturition may indicate that a prolactin-like hormone is produced by the placenta. Mammary growth, like maintenance of the corpus luteum, is a complex process, requiring the synergism of many hormones (Deansely, 1966), and with variations in requirements from species to species (Cowie et al, 1971). Experimental studies on mammary growth have shown that there is little or no mammary gland response to ovarian steroids in the absence of the anterior pituitary, but both adrenal and ovarian steroids act synergistically with prolactin and growth hormone to produce lobulo-alveolar mammary development. A placental lactogen which has both mammotropic and somatotropic activities may therefore be acting both directly as a mammotropic hormone and indirectly to stimulate the corpus

luteum.

(ii) Implantation or Culture of Placenta:

The implantation of the placenta into a recipient of the same species may provide evidence of its endocrine function. Autografts of placenta onto the intestinal mesenteric tissue of rats hysterectomized at midterm maintained mammary gland development (Mayer et al, 1950), while grafts of mouse placenta over the mammary gland of virgin female mice primed with estrogen and progesterone had mammotrophic effects (Kohmoto et al, 1970). Similarly, if placenta is cultured in vitro products of its secretion may be detected in the culture medium or via their effects on other tissues such as mammary gland cultured in the same dish (co-culture). This technique has been used to detect placental lactogen production by mouse and ruminant placentae.

(iii) Assay of Plasma from Pregnant Females:

It may be possible to detect hormonal activities in the plasma of pregnant females which are not present in males or nonpregnant females. The demonstration that such activity persists after hypophysectomy or upon the death or removal of the fetus, but not if the placentas are removed, suggests a placental origin. Luteotropic activity has been detected in the plasma of rats at midpregnancy (Matthies,1965,1967,1971; Cohen et al,1969).

(iv) Immunological Methods:

hPL shows partial immunological cross reactivity with hGH and the finding greatly assisted early studies on the hormone (Josimovich and MacLaren, 1962). The placental lactogen of the rhesus monkey is clearly a very similar protein (Shome et al. 1971). Hence, using a radioimmunoassay for lactogen or growth hormone, one could test whether placental extracts from other species cross-react in the assay, and if positive suggest that the presence of a substance immunologically similar to the hormonal standard used was present.

Using a haemagglutination inhibition method, Gusdon et al (1970) found that there is cross-reaction between hPL and placental proteins of rhesus monkey, rat, dog, pig, horse, sheep, cow and rabbit. Furthermore, once antibodies have been prepared to placental lactogen or a suitable cross-reacting antigen, the antibodies can be used to identify the site of hormone production. In the case of the human (Sciarra et al, 1963, Currie et al, 1966; Beck et al, 1969; Ikonicoff et al, 1976a) and monkey (Currie et al, 1966, Iknoicoff et al, 1971b) placentas, it is the cytoplasm of the syncytiotrophoblast.

(v) Radioreceptorassays for Prolactin (RRA-PRL) and for Growth Hormone (RRA-GH):

The development of radioreceptorassays for peptide hormones are based on the principle that in order for a hormone (peptide or protein) to exert its biological effects

binding of that hormone to its target cell is necessary (Roth, 1973). Thus, specific target cell membranes were isolated from animal tissues and used to assay for specific hormones. The advantages of these assays are that they are relatively simple to perform, quite sensitive (10 ng/ml without serum and 50ng/ml for serum samples), and most importantly, they are not species specific. For example, a radioreceptorassay for prolactin (Shiu et al, 1973) can be used to measure prolactin and placental lactogens derived from many species, whereas the conventional radioimmunoassay (RIA) is generally species specific. Of course, in any determination the RRA cannot distinguish pituitary prolactin (PRL) from placental lactogen or primate pituitary growth hormone (GH).

Fortunately, however in most species, serum levels of pituitary prolactin or growth hormone during pregnancy are low as compared to placental lactogen levels. To determine the exact contribution of the pituitary hormone level to total activity it is necessary to employ a specific radioimmunoassay. The difference between total RRA and RIA-pituitary hormone concentration represents serum concentration of placental lactogen.

Thus, by employing these two radioreceptorassays (RRA-PRL and RRA-GH), several non-primate placental lactogens have been detected and isolated in the past 3 to 4 years (Shiu et al, 1973; Kelly et al, 1974a and b; Robertson et al,

1974; Fellows et al, 1974; Handwerger et al, 1974; Martal et al, 1975; Bolander et al, 1976; Chan et al, 1976). In our laboratory, we have identified and quantitated placental lactogen concentrations in the circulation of human, monkey, rat, mice, hamster, guinea pig, cow, goat and sheep using these assays (Kelly, 1976).

Order Lagomorpha (Rabbits):

Pregnancy is terminated at any stage by hypophysectomy or by ovariectomy in the rabbit. The pituitary complex required to maintain luteal function has been studied in females hypophysectomized on day 7 and 8 of pregnancy and treated with hormones for a further 7 or 8 days (Spies et al, 1968). FSH, prolactin and possibly also LH are required, although these hormones at the doses used did not support normal fetuses. Thus, it appears that the placenta alone cannot provide an adequate luteotropic stimulus during pregnancy.

There is very little quantitative information available on mammary gland development in rabbits hypophysectomized during pregnancy. In hypophysectomized pregnant females maintained on either progesterone (Robson, 1936) or estrogen (Robson, 1937, 1939), the mammary gland is not so well developed as in normal pregnancy, but it apparently does not completely regress. This may indicate some placental mammotropin (placental lactogen) secretion, but since estrogen and progesterone can produced limited mammary gland

development in hypophysectomized non-pregnant rabbits (Cowie, 1971), further studies are required before firm conclusions can be drawn.

Using a haemagglutination-inhibition method, cross-reaction between hPL and placental protein of rabbit have been detected (Gusdon et al, 1970). However, in this method, rather large quantities of proteins were required to give inhibition, and non-specific effects cannot be excluded. Thus this aspect warrants further investigation using other techniques.

However, studies by Forsyth (1975) demonstrated that when rabbit placentas were cultured with mouse mammary gland explants, she found that the rabbit mammary lobuloalveolar tissue did not exhibit a lactogenic effect.

Furthermore, using radioreceptorassays for prolactin, we could not demonstrate any lactogenic activity in rabbit placental extracts (Kelly et al, 1976; McNeilly et al, 1978), and this is substantiated by another group in France (Durand et al, 1977) using the same assay. Similarly, using a radioreceptorassay for placental lactogen, I could not show any significant placental lactogen activity from rabbit placental extracts.

Order Rodentia (rat, mice, role, hamster and guinea pig):

(i) Rat:

Lyons in 1944 showed that rat placental extracts are capable of synergizing with ovarian steroids to induce mammary lobulo-alveooar growth and also early indication of lactation. Later Ray et al (1955) demonstrated by means of assay of its various components that the most potent mammotropic activity was found in the trophoblastic tissue from day 12 placenta.

In rats, removal of fetuses plus fetal and placental tissue on day 12 or 16 of pregnancy reduced the weight of the mammary glands to control, non-pregnant levels by day 21 (Desjardin et al, 1968). Removal of the fetus alone on day 16 had no significant effect on mammary weight, DNA, or RNA content on day 21. Removal of the fetus on day 12 depressed all these parameters to some extent but considerable mammary development had nevertheless occurred between day 12 and 21. The placenta, therefore, appears to make some hormonal contributions to mammary development before mid-pregnancy and provides the major stimulus in the second half of pregnancy.

Current investigation using radioreceptorassays for prolactin (RRA-PRL) by Shiu et al (1973) showed that two peaks of serum rat placental lactogen (rPL) were observed during pregnancy. The first peak of rPL occurred at mid-pregnancy and the second peak occurred near term. Upon Sephadex G-100 fractionation of day 17 pregnancy rat serum, all the lactogenic activity is found in fractions

with a molecular weight of 22,000 (Kelly et al, 1974). Furthermore, the half-time disappearance rate of rPL from day 12 and day 19 were also different. rPL from day 12 was 19.5 minutes whereas from day 19 was 1.2 minutes.

In 1974 Robertson and Friesen partially purified rPL from rat placentas. The molecular weight of rat placental lactogen is approximately 18,000 as determined by gel filtration on Sephadex G-100, and 22,000 M.W. as determined by electrophoresis upon sodium dodecyl sulfate (SDS)-polyacrylamide gels. The isoelectric point of rat placental lactogen is between pH 6.5 and 7.0 as determined by isoelectric focusing. The amino acid composition and primary structure have not been determined.

When the partially purified rPL was examined by RRA-PRL and RRA-GH, it proved 41% as active as ovine prolactin standard (NIH-S-P-10, 25 IU/mg) but 169% as active as human placental lactogen preparation in the RRA-PRL. In the RRA-GH, the rPL has minimal growth hormone-like activity.

Guinea Pig:

Hypophysectomy of the guinea pig on day 24 to 26 of pregnancy leads to resorption of the young within 2 days. However, by 40 days pregnancy can continue independently of the pituitary and viable young are born at term, but the mammary glands were smaller than usual (Pencharz et al,1934). Thus, it is suggested that guinea pig placenta may secrete mammotropic substances during pregnancy.

Using radioreceptor assays for prolactin (RRA-PRL) and for growth hormone (RRA-GH), it is found that the peak level of guinea pig placental lactogen occurred at 50-55 days. Furthermore, the relative ratio of prolactin to growth hormone activity of guinea pig serum placental lactogen is 1:2 (50-58 days of gestation). Gel-filtration studies on guinea pig extracts and serum demonstrated that the guinea pig placental lactogen is approximately 22,000. Until now, guinea pig placental lactogen has not been purified.

Hamster:

The first report to suggest that hamster placenta might secrete placental lactogen is by Talamantes (1973). The existence of such a hormone is further substantiated by the finding using RRA-PRL and RRA-GH to measure the placental lactogen levels in the serum and in the placental extracts of hamsters. Like guinea pigs, serum level of hamster placental lactogen reaches its peak activity before parturition about day 10-12 (gestational period is 15), and declines before parturition (Kelly et al,1976). Molecular weight determination by gel filtration in Sephadex G-100 indicates that it has a molecular weight of 18,000 to 23,000. Hamster placental lactogen has not been purified yet.

Order Carnivora (dog, fox, cat, mink and ferret):

Dogs are dependent upon the ovary for the maintenance of pregnancy for most, if not all, of gestation. Sokolowski (1971) found that ovariectomy in the dog as late as day 56 postbreeding resulted in premature termination of gestation (gestational period is 59-68 days). An enhancement of luteal function has been reported for the pregnant dog as compared to the non-pregnant dog beginning at about 15 days post-ovulation (Smith et al, 1974). The increase in progesterone secretion suggests the possibility of a placental luteotropin as implantation begins at about this time (Holst et al, 1971). Other workers, however, have not found an enhancement of luteal activity in the pregnant dog (Concannon et al, 1975; Hadley, 1975).

Using a haemagglutinin-inhibition method, Gudson et al (1970) reported that there might be a placental lactogen in the dog placenta which cross-reacts immunologically with hPL. In our own studies (Kelly et al, 1976) we found that dog placental extract cross-reacted in the radioreceptor-assay for prolactin using rabbit mammary gland as receptors (Kelly et al, 1976) and in the radioreceptorassay for placental lactogen using sheep liver as receptors.

However, further studies are required to substantiate our claims.

We have not yet examined the placentas of cat, fox, mink and ferret.

Order Perissodactyla (Horse and Asses):

There is no evidence to indicate the existence of horse placental lactogen. In my own studies, I could not find any cross-reactivity between horse placental extracts and ovine placental lactogen in the radioreceptor-assay for placental lactogen using sheep liver as receptors.

Order Artiodactyla (pig, camel, llama, cow, goat, sheep, deer):

This order is actually divided into three suborders, the Suiformes (pig), the Tylopoda (camel and llama) and the Ruminantia (cow, goat, sheep and the deer).

Because most of the information accumulated in recent years is concerned with placental lactogens in the cow, goat, and the sheep, therefore, I would like to confine my discussion to these three species.

(i) Cow:

Gusdon et al (1970) are the first to report the possibility of bPL in bovine placenta. They demonstrated that the extracts of term bovine placental extracts cross-reacted with anti-hPL serum. Later, using co-culture of fragments of cotyledons with mouse mammary gland explants, Forsyth and co-workers (Forsyth and Buttle, 1972; Forsyth, 1973; Buttle and Forsyth, 1976) demonstrated that placental lactogen is secreted in cows on days 36, 178, 182 and 270 of pregnancy. Bovine placental lactogen originated from fetal cotyledon and showed no detectable

cross-reaction in immunoassays for bovine prolactin and growth hormone.

Matthies (1974) also reported that presence of placental lactogen in placental extracts when they were injected directly into the rat mammary gland causing proliferation of mammary gland.

Subsequently, several investigators confirmed the presence of placental lactogen in the cow by using a RRA-PRL and RRA-GH to detect bovine placental lactogen (Kelly et al, 1976) Unlike the secretion of monkey and human placental lactogen, Kelly et al (1976) found that the levels of bovine placental lactogen remained fairly constant throughout pregnancy. The low levels of bovine placental lactogen was unexpected. The reason for this is still not clear. It is possible that a low secretion rate in vivo or rapid clearance of hormone from blood might result in undetectable levels of placental lactogen in the peripheral circulation. Upon gel-filtration of serum and placental extracts on Sephadex-G-100, it appears that the molecular weight of bPL is approximately 40,000 to 50,000.

Recently, Bolander et al (1975, 1976) have reported that they have purified bPL from bovine placental cotyledons with 40-fold purification. When the growth promoting activity of their bPL was tested in hypophysectomized rats, it was 5% as potent as bovine growth hormone standard. Furthermore, when their bPL was tested for its lactogenic

activity in the N-acetyl-lactosamine (NAL) synthetase assay, it exhibited approximately 4.5% of the potency of bovine prolactin standard.

The molecular weight estimated by Bolander et al is 22,150 daltons with an isoelectric point is 5.9. The amino acid composition of bPL closely resembled that of bGH and bPRL except for a higher content of serine and glycine and a lower leucine content. By Outchlerlony immunodiffusion, bPL forms lines of partial identity with bGH against bGH antisera and with ovine placental lactogen (oPL) against oPL antisera.

With the purified bPL, Bolander et al (1976) have developed a specific radioimmunoassay for bPL. Using this RIA, they have quantitated the levels of bPL in the circulation of beef and dairy cows. In both dairy and beef cows, serum bPL levels remain low (less 50 ng/ml) during the first two trimesters and then rise rapidly between 160-200 days of gestation to reach a plateau. The bPL levels do not decline prior to parturition. During the last trimester, serum levels in dairy cows, 1103±342 ng/ml, are significantly higher than those in beef cattle, 650±137 ng/ml; furthermore, dairy cows with a higher milk production also tend to have higher bPL levels. Serum levels are almost twice as high in twin pregnancies and are not correlated with fetal sex or birth weight. The bPL levels in the milk and amniotic fluid from dairy cattle during the last trimester are approximately 86% and 25% of the serum values, respectively,

suggesting that bPL enters these fluids by passive diffusion.

At present,it is not clear why a large discrepancy exists between the reports of Bolander et al (1976a,b) and others (Buttle et al,1976; Kelly et al,1976), in respect to the secretion pattern of bPL and the molecular weight of bPL. One possible explanations may be that the hormone preparation obtained by Bolander et al is not pure. In most cases of the purification of placental hormones, such as hPL, mPL and oPL, it requires more than a 1000-fold purification before one can achieve 99% purity. In any case, the resolution of the discrepancy awaits the purification and characterization of bPL by other groups.

(ii) Goat:

The goat is a spontaneous ovulator with an inter estrus interval of 20 to 21 days. The integrity of the pituitary is essential for the maintenance of pregnancy in this species. In 1963, Cowie et al reported that hypophysectomy in pregnant goats causes abortion at any stage of pregnancy. Ovariectomy at any stage of pregnancy also causes abortion in the goat (Drummond et al, 1962; Meites et al, 1961). Since hypophysectomy in the goat will lead to abortion it has been concluded that the conceptus does not produce a luteotropic factor, but this may be a matter of degree. It has been noted by Van Rensburg (1971) that after an initial decrease during the first 40 days of pregnancy the luteal cells of the corpus luteum of the pregnant goat decrease in size and then increase until day 110 before a slow decline to parturition sets in. The secretory activity of the corpus luteum tends to follow the change in the size of the luteal cells with peripheral plasma progesterone concentrations reaching their maximum about day 110. Therefore, it is possible that the increase in size of the luteal cells over the period 40-110 days may arise through stimulation by some placental luteotropins.

The growth and differentiation of the mammary gland during pregnancy is a response to the action of many hormones (Cowie and Tindal, 1971), one of which is prolactin. This hormone has been considered to exert a major influence as a mammotropin. However, Buttle et al (1972) and Forsyth

(1972) reported that when goat plasma samples taken during pregnancy were examined for prolactin by radioimmunoassay and for total lactogenic activity by a rabbit mammary gland culture assay, high levels of lactogenic activity were detected in the second and last third of pregnancy when the concentration of immunoreactive pituitary prolactin was low or even absent. Co-culture experiments (Forsyth, 1972) demonstrated that this mammotropic substance (caprine placental lactogen, cPL) is secreted by fetal cotyledons, and could be detected in the maternal circulation from the 9th week (64 days) of gestation until term. However, it is not clear which cells of the placental cotyledons are secreting placental lactogen and also it is not clear at what stages of pregnancy this hormone is first secreted.

Using radioreceptorassay for prolactin (RRA-PRL) and RRA-GH, Kelly et al (1976) and Currie et al (1977) estimated the serum levels of caprine placental lactogen (cPL) throughout pregnancy. In their studies, they found that lactogenic and GH-like activities increased from less than 100 ng/ml (ovine prolactin and growth hormone equivalents) about 60 days after mating to reach peak levels (400-1600 ng/ml) between days 110 and 130 of pregnancy. The levels of both activities increased in essentially the same fashion but during the last 15 days of pregnancy, lactogenic activity declined less than GH-like activity. This probably reflected the increased secretion of pituitary prolactin near parturition.

When serum from a pregnant goat or a simple alkaline extract of placental cotyledons was fractionated on Sephadex G-100, lactogenic and GH-like activities eluted with a molecular weight of 20,000 to 22,000.

More recently, Grissom et al (1977) reported that they have purified caprine placental lactogen from goat placentas. They found that cPL has an isoelectric point of 6.4 and its molecular weight estimated by gel-filtration was 22,000 daltons. The amino acid composition of cPL resembles that of ovine and bovine placental lactogens. Furthermore, cPL is as potent as ovine prolactin when tested in the radioreceptorassay for prolactin.

(iii) Sheep:

Although there is no compelling evidence to demonstrate the existence of ovine chorionic gonadotropin, indirect evidence has accumulated to suggest that there is a luteotropin secreted by the conceptus, possibly oPL. For the continuation of pregnancy in the ewe, the presence of an actively secreting corpus luteum is essential during the first 50-60 days. Ovariectomy, enucleation of the corpus luteum, or hypophysectomy, during this period will lead to the termination of the pregnancy. After day 50-60 the activity of the corpus luteum is maintained following hypophysectomy, which suggests that the conceptus may be producing a luteotropin that not only maintains the functional activity of the corpus luteum but may also stimulate increased progesterone synthesis and secretion by the conceptus. However, it is not yet clear whether the putative luteotropin is a distinct hormone or ovine placental lactogen (oPL).

One of the early pieces of evidence that suggested ovine placentas might secrete a mammotropin was a report by Denamur and Martinet (1961). They found that hypophysectomy of pregnant ewes after 50 days of gestation did not affect the normal course of pregnancy and the development of the mammary gland, although the mammary gland was not so developed as in intact animals. Furthermore, transient lactation also took place in these hypophysectomized ewes.

Gusdon et al (1970)using haemagglutination inhibition test also reported that crude ovine placental extracts cross-react with anti-hPL antiserum. Later, Forsyth (1974) confirmed the presence of placental lactogen in the sheep by using co-culture experiments.

Using radioreceptorassays for prolactin and for growth hormone, Kelly et al (1974) were the first to quantitate the plasma levels of oPL in the sheep during pregnancy. By day 60 of gestation and thereafter placental lactogen concentrations increase as pregnancy advances, reaching peak concentrations of 1 to 2 µg/ml (oPRL equivalent in the RRA-PRL) on days 95 to 114 of gestation. After the initial peak there is generally a decline in placental lactogen concentration followed by another peak before parturition oPL concentrations slowly declined from approximately 1 µg/ml to less than 500 ng/ml by 12 hours before parturition and then decreased quite rapidly post-partum. By re-assaying the serum samples during pregnancy with a radioreceptorassay for growth hormone (RRA-GH), similar results were obtained. However, the serum concentration of growth hormone-like activity is much lower than prolactin-like activity, the ratio of PRL/GH is approximately 3 or 5 to 1, whereas in placental extracts, the ratio of PRL/GH activity is about 1:1. This may suggest that serum contains substances that interfere in the RRA-PRL more than with RRA-GH. oPL was demonstrated (assayed by RRA-PRL) in the trophoblasts as early as day 16-17 of pregnancy

(Martal et al,1977). Concentrations in the placenta rise slowly until about day 100 when there is a rapid increase to reach 70 ug of prolactin equivalent /g fresh placental tissue and remain at that level. After 140 days of gestation, the placental content decreased.

(a) Chemistry of oPL:

In the last few years, oPL has been purified by several laboratories (Handwerger et al,1974; Martal et al,1975: Chan et al, 1976). The molecular weight of oPL appears to be approximately 22,000 daltons. The isoelectric point of oPL determined by Handwerger et al (1974) is approximately 6.8, whereas in our own studies, the isoelectric point of oPL is approximately 8.8. The amino acid composition of oPL is similar to ovine growth hormone (oGH) and oPRL except for a lower content of leucine and phenyalanine (Hurley et al, 1976). It consists of 192 amino acids residues. Like oPRL, but in contrast to oGH, oPL contains 6 half-cystine and 2 tryptophan residues while its COOH-terminal sequence is the same as that of oGH: Cys-Ala-Phe-OH. The primary structure of oPL has not yet been defined. Partial characterization of the cyanogen bromide fragments of oPL by Hurley et al (1976) suggests that oPL is very similar to oPRL and oGH.

(b) Cell-origin of oPL:

Using an immunofluorescent method, Martal et al(1977) demonstrated that oPL is located in large cells of the monostratified epithelium of chorionic villi. These cells are both mono- and binucleated and PAS-positive.

(c) Immunological Properties of oPL:

Handwerger et al(1974) reported that oPL-antiserum cross-reacts with ovine growth hormone but there is no cross-reaction with human placental lactogen, human prolactin, human growth hormone, and or ovine prolactin by Ouchterlony diffusion method. In subsequent studies by the same group(Handwerger et al,1977), they acknowleged that their previous observations may be erroneous, and confirmed that oPL-antiserum does not cross-react with any hormone preparations.

(d) Secretion and Distribution of oPL measured by RIA:

Handwerger et al (1977) reported that by RIA, oPL was first detected in the maternal plasma at 41-50 days of gestation and reached a peak concentration of 1.5 to 2.5 ug/ml at 121-131 days of gestation. The oPL concentration in plasma was 336 ng/ml and in allantoic fluid was 29 ng/ml. After surgical removal of the placenta, oPL disappeared from maternal plasma with a half-life of 29 minutes.

(e) Biological Effects:

oPL is a potent lactogen which stimulates lactation in vivo in the rabbit and mouse mammary gland explants (Handwerger et al,1974; Martal et al,1975; Chan et al,1976) and also is a potent growth promoting protein as demonstrated in hypophysectomized rats (Handwerger et al,1974; Chan et al,1976).

Handwerger et al(1975) injected partially purified oPL into fasted pregnant and non-pregnant ewes and found that intra-

venous administration of PL produced an acute decrease in plasma fatty acid, glucose and amino nitrogen concentrations. The effect of oPL on FFA concentrations is opposite to the diabetogenic, anti-insulin effects of hPL and hGH administration in human (Grumbach et al,1968; Beck et al,1967), and also opposite to the effects of oGH administration into sheep (Manns et al,1965).

In summary, only hCG and hPL are structurally defined, whereas placental protein hormones from other species are not. hCG is shown to play an important role in stimulating the corpus luteum to synthesize and to secrete progesterone, but the role played by hPL during pregnancy is not clear. Until now, there is no compelling evidence for the existence of non-primate chorionic gonadotropin with the exception of pregnant mare serum gonadotropin (PMSG). With the development of radioreceptor assays for prolactin and for growth hormone, we are able to detect and to quantitate placental lactogens in the circulation and in the placenta of sheep. goat, cow, rat, mouse, guinea pig, and hamster. I have been engaged in the purification of oPL and subsequently on the studies of oPL in collaboration with Dr. H.A. Robertson's group at Ottawa. Right now, I wish to review some aspects of the reproductive biology of sheep before I present my own studies on oPL.

SECTION II : OVINE REPRODUCTIVE PHYSIOLOGY DURING PREGNANCY

In this section I wish to discuss some aspects of ovine reproductive physiology especially that concerned with pregnancy. This discussion hopefully will provide a background for the understanding of possible roles played by ovine placental lactogen during pregnancy. This discussion will be divided into three subtopics; they are: (i) biology of ovine placenta, (ii) hormonal patterns in the maternal blood during pregnancy, and finally (iii) some possible mechanisms for maintaining corpus luteum function during pregnancy.

(i) Biology of Ovine Placenta:

Following ovulation the ovum passes into the fallopian tube aided by the movement of the fimbriae of the infundibulum. Fertilization occurs (in the lower region of the ampulla of the oviduct) within hours after ovulation. When the embryo has reached the morula stage, on day 4, i.e. 72 hours after ovulation, it passes through the uterotubal junction. From the 5th day it undergoes rapid elongation and by the 17th day extends throughout the length of both uterine cornua. The allantois grows out as a bifid sac from the caudal end of the embryo and elongates rapidly within the chorionic sac, from which it is separated by the extraembryonic coelom. Fusion of the allantoic mesoderm with that of the chorionic sac is complete by about the 22nd day at which time the vascular fetal cotyledons make their appearance.

The wall of the chorionic sac becomes sticky about the 17th day and adheres to the convex outer surface of the maternal caruncles, which have circular elevations on the endometrium, often pigmented.

Modification of the cytological character of the maternal caruncular epithelium is first apparent on the 16th day of pregnancy. A reduction in the number of epithelial cells is usually associated with nuclear vacuolation, swelling and disintegration. The subepithelial stroma becomes oedematous and contains increased number of eosinophils.

The structural appearance of the chorionic sac at day 17 to 22 is a simple or pseudostratified cuboidal epithelium. Pleomorphism of the epithelium is apparent and is probably related to the differentiation of the epithelium into two cell types: the uninucleate cuboidal trophoblast, and the rounded binucleate giant cells.

The cuboidal epithelial cells constitute about four-fifths of the trophoblast cell population and possess a large irregularly-shaped nucleus with a single large nucleolus and finely dispersed chromatin. Their cytoplasm is moderately dense and contains, predominantly in the apical part of the cell, ovoid to elongated mitochondrial profiles with a dense matrix and moderate numbers of lamellar cristae. The Golgi bodies are small and are located laterally in the cytoplasm of the cell. Adjacent lateral plasma membranes interweave in a complex manner in some areas and exhibit infrequent desmosomes and varying degrees of separation. Close to the maternal tissue these membranes posses numerous microvilli interdigitating with corresponding maternal microvilli. The cuboidal cells can further divide into two types. At one extreme are the majority of the cells,

which contain, as well as the organelles described above, elongated cisternae of granular endoplasmic reticulum located in the lateral regions of the cells. At the other, are small numbers of cells, usually arranged in clumps, containing fewer shorter cisternae of granular endoplasmic reticulum and numerous darkly-staining granules, which vary in size, are pleomorphic in form, and appear in some cells to be undergoing degenerative changes. Large granules may distort the nucleus. Between these two extreme cell types are found intermediate forms. At 70 days of gestation , only one form of cuboidal epithelial cell is present. It differs from those described above in having numerous free ribosomes, short dilated cisternae of agranular endoplasmic reticulum. Ocassionally a small Golgi complex is seen, but neither the darkly-staining granules nor the elongated cisternae of granular endoplasmic reticulum characteristic of the mature cuboidal cells are present.

The binucleate giant cells, which constitute about one-fifth of the total trophoblast tissue, are found, usually singly, among the cuboidal epithelial cells. These cells neither rest on the basement membrane nor contribute to the fetal-maternal junction. Their two large rounded, nuclei have one or two small nucleoli and clumped chromatin. Mitochondria, similar to those of the cuboidal epithelium, are spread throughout the cytoplasm, although they usually are less numerous in the lateral regions of the cells. The cytoplasm which is denser than that of the cuboidal cells, contains numerous short, dilated cisternae of granular endoplasmic reticulum, and a large, complex Golgi apparatus. The plasma membrane of these cells does not show the complex interweaving or obvious desmosomes typical of the cuboidal cells. The binucleate

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cells usually contain either numerous membrane-bound particles of varying osmophilia or numerous rosettes of glycogen. Binucleate cells in the 70 day trophoblast exhibit polarity; majority of their mitochondria and a small but often complex Golgi body are situated in that part of the cell nearest to the maternal tissues. Small clear, membrane-bound vesicles are scattered throughout the cytoplasm, and some cells are, in addition, filled with multivesicular bodies of varying size and density. Mitochondria with tubular or vesicular cristae, and numerous dilated irregular cisternae and tubules of endoplasmic reticulum filled with a flocculent material of varying density, are also present in the cytoplasm. Although cells containing densely osmophilic membrane-bound particles like those of the older cells are not present at the 70th day there are a few glycogen-containing cells. The binucleate trophoblasts are identified by their PAS-positive properties.

The first signs of the interdigitation (implantation) or embryonic and maternal tissues appear early in the 4th week (28 days) of gestation. A series of ridges and grooves in which there is a close association between the chorionic and the endometrium develops on the maternal caruncular surface. These endometrial grooves, which deepen and branch as development proceeds, are lined in some area by flattened multinucleated syncytial mass, while other areas appear to have an attenuated cytoplasmic covering. The syncytial mass was normally PAS-positive in appearance, although not as strongly as the binucleate cells located within the trophoblast.

At all stages the chorionic villi contain vascularized mesenchyme and are completely covered by cuboidal uninucleate trophoblastic

epithelium and binucleate cells. During the 5th and 6th weeks (35 to 42 days) of development, the binucleate cells are the predominant cell type in the apical regions of the chorionic villi.

An important feature of the chorionic villi of the placentome in the later stages of gestation (after the 100 days) is the invasion of the columnar trophoblast by chorio-allantoic capillaries. In this process, which is not observed at any stage in the intercotyledonary zone, the capillaries become deeply invaginated into the chorionic epithelium whose cells are intimately molded on to them.

The placentomes are 60 to 100 in number in the sheep but show great variation in size and shape. The placentomes of the sheep increase in size and number up to about the 90th day after which they shrink with some possible decrease in number.

The intercotyledonary zone of the sheep placenta comprises the remaining part of the chorionic sac not involved in the formation of the placentomes. It shows regional thickenings in relation to the pits which receive the mouths of the uterine gland; these are so-called "areolae" in which the chorionic epithelium is specialized for absorption.

The length of gestational period in the sheep is 145-155 days.

The above accounts are based on the works of Wimsalt(1951), Amoroso(1952), Davies(1952), Asdell(1946), Davies et al(1966), Bjorkman (1965), Boshier(1969), and Boshier et al(1977).

(ii) Hormonal Patterns in Maternal Blood during Pregnancy:

(a) Progesterone

During the estrus, serum values varied from 0.12 ng/ml (equivalent to anestrus) to 2.0 ng/ml from day 10 to 14. After mating, pregnant and

non-pregnant values did not differ till day 16, when values rise in the pregnant ewes to 2.5 ng/ml at day 50, reach a plateau at days 125-130 (12-20 ng/ml), finally exhibit a steep fall on the day of lambing. Appreciable amounts are still present at the beginning of parturition, falling below 1 ng/ml at the end of the birth process (Bassett et al, 1969; Stabenfeldt et al,1972; Thompson et al,1974b).

(b) Estrogen:

Few determinations are reported with the exception of the situation at term. Pregnancy levels are low, remaining less than 50 pg/ml for most of the period and rising to 100 pg/ml just 1 day before birth. On the day of parturition, mean levels rose to 400 pg/ml, then fell to 50 pg/ml in less than 24 hours (Thompson et al,1974b). Challis (1971) first reported this estrogen peak in sheep and noted the terminal increase in the estrogen/progesterone ratio.

(c) Corticosteroids:

Plasma corticosteroids rose in control and ovariectomized pregnant ewes 72-78 hours prepartum (Thompson et al,1974a). At 3 to 8 days prior to parturition, the corticosteroid levels were 11 to 19 ng/ml, rose and reached a plateau at 25-35 ng/ml 24 to 48 hours before parturition. Over the same time interval, adrenalectomized sheep showed values of 3 to 6 ng/ml.

(d) Luteinizing Hormone, LH:

Cycling ewes showed baseline levels of 2 to 3 ng/ml and peaks related to estrus of 30 to 200 ng/ml(Geschwind et al,1968;Goding et al,1973; Scaramuzzi et al,1970).Values dropped to < 1 ng/ml in observations

which continued to the twentieth day of pregnancy (Niswender et al, 1968). At days 123-128, no maternal blood LH was detected (Foster et al, 1972).

(e) Follicle-Stimulating Hormone, FSH:

The only figures found for FSH in pregnancy were those of Chamley et al (1974). To the nearest integer, values were: day 42, 34 ng/ml; day 91, 72 ng/ml; day 126, 59 ng/ml; day 147 (parturition), 35 ng/ml.

(f) Prolactin:

Levels in the blood of pregnant ewes ranged between 20 and 80 ng/ml during the first 20 days. At 2 days prepartum a sharp rise began, reaching 400 ng/ml on the day of parturition with wide but smaller fluctuations for 8 days (Davies et al, 1971). Comparable results showed 20-40 ng/ml up to 80 days, an increase from 50 to 100 ng/ml within 10 days of parturition, and a terminal peak of 100 ng/ml representing a mean for seven ewes (Kann et al, 1974).

(iii) Possible Mechanisms for Maintenance of Corpus Lutum Function in Early Pregnancy:

1. It has been shown that when embryos of ewes are transferred to synchronous recipients at any time up to day 12, pregnancy can ensue; whereas, when they are transferred after this time, the corpus luteum is not maintained beyond day 15, (ie. the time of luteal regression in the normal estrous cycle) and consequently, pregnancy does not follow (Rowson et al, 1967). Thus we can say that the maternal organism recognizes the presence of an embryo by, or before, day 12 and by this time

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initiates some mechanism preventing luteolysis on day 15. The nature of this antiluteolytic factor is not known.

For the continuation of pregnancy in the ewe, the presence of an actively secreting corpus luteum is essential during the first 50-60 days. Ovariectomy, enucleation of the corpus luteum, or hypophysectomy, during this period will lead to the termination of the pregnancy. After 50-60 days the activity of the corpus luteum is maintained following hypophysectomy, which suggests that the conceptus may be producing a substance that not only maintains the functional activity of the corpus luteum but may also enhance the increased progesterone synthesis and secretion by the corpus luteum.

2. The corpus luteum may be maintained by a gonadotropin either of pituitary or embryonic origin. However, there is no evidence for an increase in radioimmunoassayable LH in early pregnancy in the ewe (Niswender et al, 1968; Goding et al, 1969). Furthermore, it is found that intra-carotid infusions of 10 µg/hr of LH will not prevent the luteolysis which results from the superimposed infusion of as little as 5 µg/hr PGF_{2a} to sheep with ovarian transplants (Cerini et al, 1973). Thus, it appears that LH alone can not maintain the CL function. Although infusion of prolactin alone also failed to prolong luteal function in the intact ewe as demonstrated by Karsch et al (1971), intraarterial prolactin infusion (100 µg/hr) plus LH prevents

luteolysis when superimposed on infusion of PGF_{2a}(5 ug/hr) (Cerini et al, 1972). Thus, these studies suggested that the CL function is maintained by both LH and prolactin.

3. The embryo may be capable of inhibiting $PGF_{2\alpha}$ secretion by the uterus. Wilson et al (1972) put forward preliminary evidence that the $PGF_{2\alpha}$ content of uterine endometrium and uterine vein blood was increased, rather than decreased, in early pregnancy. However, these data were obtained by bioassay and require confirmation. Nevertheless, it is possible that embryo may be able to prevent secreted $PGF_{2\alpha}$ gaining access to the ovary.

SECTION IV: OBJECTIVES OF THE PRESENT INVESTIGATIONS

The objectives of the present studies are several fold. They are:

1. To establish a method for the purification of ovine placental lactogen (oPL) from ovine placental cotyledons in sufficient purity and quantity for chemical and biological studies.

2. To search for and characterize the receptor sites for oPL in ovine tissues in order to locate possible sites of action for oPL in the sheep during pregnancy.

3. To develop a sensitive radioimmunoassay for oPL that can measure oPL specifically and quantitatively.

4. To study the secretion of ovine placental lactogen in the sheep.

5. To demonstrate the biosynthesis of oPL.

6. To study the biological effects of oPL in rats.

SECTION IV: METHODS AND MATERIALS

A. PURIFICATION AND CHARACTERIZATION OF OVINE PLACENTAL LACTOGEN(oPL)

Assay for monitoring hormonal activity of oPL- Radioreceptorassay for growth hormone-like activity(RRA-GH).

A radioreceptorassay for measuring growth hormone or growth hormone-like activity utilizing rabbit liver receptors as described by Tsushima et al (1973) with slight modifications was employed to detect the hormonal activity of ovine placental lactogen during purification.

Method of isolation of specific receptors:

Rabbit liver obtained from mid or late pregnant rabbits was cut into small fragments before being homogenized in 5 volumes of 0.3 M sucrose solution. Homogenization was carried out at 4 C using Polytron Pt-10 (Brinkmann) for one minute with the dial set at maximum. The homogenate was filtered twice, first with 4 layers and then 8 layers of cheesecloth. The filtrate was centrifuged at 780 x g for 20 min at 4 C, the supernatant was centrifuged at 15,000 x g for 20 min, and the pellet was discarded. The supernatant was again centrifuged at 100,000 x g for 45 min to obtain the microsomal pellet which contains most of the broken cell membrane. Of the total binding activity, 70-80 % was recovered in this fraction. The microsomal pellet was suspended in 0.025M Tris-HCl, pH 7.6 containing 10 mM $MgCl_2$; and kept frozen at -20 C. When required for assay the frozen receptor was thawed, homogenized in a glass homogenizer with an

appropriate volume of Tris-HCl buffer such that the protein concentration in 0.1 ml volume of the suspension contained 100 to 200 ug of protein as determined by the Lowry procedure (Lowry et al,1951). Membrane suspensions prepared from one rabbit liver tissue provides sufficient receptors for 6,000 tubes .

2. Iodination procedure for hormone preparation(human growth hormone, hGH,NIH-HS 2019G, 2.2IU/mg) for RRA-GH:

[^{125}I]iodo-hGH was prepared by the lactoperoxidase enzyme method of Thorell and Johannson(1971), using 1 mCi of Na^{125}I(New England Nuclear), 5 ug of hGH, 5 ug of lactoperoxidase, 5 ul of 30% hydrogen peroxide at 1:15000dilution, and 25 ul of 0.05M phosphate buffer, pH7.4 in a final volume of 85 ul. At the end of one minute chemical reaction, 1 to 2 ml of 0.025 M Tris-HCl, pH 7.6 was added immediately to the reaction tube after 5 ul of the reaction mixture was taken out for specific activity determination. Unreacted iodide and damaged hormone were separated from intact iodinated hormone by gel filtration on Sephadex G-100 column(1.5 X 50 cm) using 0.025 M Tris-HCl,pH 7.6 as eluting buffer. The Sephadex G-100 column was pre-treated at once with 1-2 ml of 0.025M Tris-HCl. pH 7.6 containing 2.5% bovine serum albumin(BSA) in w/v in order to minimize the loss of iodinated proteins.

In order to determine the specific activity of iodinated hormone, 5 ul of the reaction mixture was removed and diluted with 1 ml of Tris-HCl buffer, pH 7.6 containing no BSA. Then 0.1 ml of 0.01 M phosphate buffered saline, pH 7.4 containing 0.1% BSA and 2 ml of 10% trichloroacetic acid

(TCA) solution were added consecutively to 0.1 ml of the diluted reaction mixture. After 3 hours of incubation at 4 C, the total radioactivity of the mixture was determined and then the tube was centrifuged at 780 x g for 20 min. The supernatant was decanted and the precipitate was counted in the LKB autogamma counter. The incorporation of the radioactivity into protein is expressed as the TCA precipitable radioactivity over the precount of the TCA reaction mixture as a percentage. The specific activity of the iodinated hormone is defined as the total precipitable counts divided by the amount of protein used for iodination(5 ug). The percentage of incorporation into hGH was 55-75%, and its specific activity was 110-130 uCi/ug of protein.

3. Incubation procedure:

All dilutions were made with 0.025M Tris-HCl buffer, pH 7.6 containing 0.1% BSA and 10 mM $CaCl_2$. The assays were carried out in glass tubes(12X75 cm) containing 200 ul of diluent, 100 ul of hormone standard (hGH), or 100 ul of known or unknown sample, and 100 ul of [^{125}I]-iodo hGH (approximately 80,000 to 100,000 cpm). During the 3 hours incubation period at room temperature, the tubes were shaken vigorously for 30 seconds every 30 min. The reaction was terminated by the addition of 3 ml ice-cold 0.025M sodium acetate buffer, pH 5.4 containing 0.1% BSA, then centrifuged at 780 x g for 20 min at 4 C. The supernatant was decanted and the membrane bound [^{125}I]-iodo hGH in the precipitate was counted in the LKB autogamma counter.

Starting Materials

Two possible sources of raw material for ovine placental lactogen purification have been examined: (1) ovine placental cotyledons removed at the time of surgery were kindly provided by Dr. Hamish A. Robertson, Reproductive Physiology, Animal Research Institute, Agriculture Canada, Ottawa, Canada; These tissues were immediately frozen and stored at -20 C without separating maternal and foetal cotyledons. (2) Ovine placental cotyledons(foetal and maternal) at mid pregnancy (60 to 120 days of gestation) obtained within 1 or 2 hours after death in the slaughter house, Auckland, New Zealand, were immediately stored frozen at -20 C. These tissues were sent to Winnipeg within 30 days after collection. Arrangements for collection and shipment were coordinated by Drs. Nicholson and Lapwood, Massey University, New Zealand.

Hormone Preparations

Human growth hormone (NIH-2019G,2.2 IU/mg), ovine growth hormone (oGH, NIH 0-9861, 2.0 IU/mg), monkey growth hormone (mGH, NIH M 945A, 0.9 IU/mg), bovine growth hormone (bGH, NIH B-1003A, 2.0 IU/mg), porcine growth hormone (pGH, NIH P-526B, 1.5 IU/mg), canine growth hormone (cGH, NIH D-100A, 1.9 IU/mg), rat growth hormone (rGH, NIH RP-1, 0.6 IU/mg), human placental lactogen (hPL, NIH, 95% electrophoretically pure), ovine prolactin (oPRL, NIH-P-S-10, 26 IU/mg), ovine luteinizing hormone (oLH, NIH-LH-S18), bovine prolactin

(bPRL, NIH-P-B3), and all other hormone preparations were kindly supplied by the NIAMDD of the NIH,USA.

Protein Measurement

Protein concentrations of the fractions collected during purification were estimated by measuring the absorbance at 278 nm, making the assumption that one unit of absorbance was equivalent to protein concentration of 1 mg/ml. For more accurate determinations, the protein content of the original crude extract and of the pools containing oPL at different stages of purification were estimated by the method of Lowry (Lowry et al,1951) using bovine serum albumin(BSA) as standard. The final purified product was weighed after lyophilization, and its protein content per unit weight was determined by spectrofluorometer (Aminco-Bowman), using ovine prolactin (NIH-P-S-10) as standard; excitation was at 278 nm and emission at 350 nm.

Concentration of volume by ultrafiltration

All concentration procedures were carried out at 4C in an Amicon Diaflo cell of various capacity (10,60, 450, and 2,000 ml). The size of cell used depended on the initial volume required to concentrate and the final volume which was desired. UM-10 membrane filters were used in all concentration steps.

Purification Procedures

All steps were carried out at 4 C unless otherwise specified.

1. Extraction:

Placental tissues which were obtained at the time of surgery or slaughter were immediately frozen at -20 C without separating maternal and foetal cotyledons. At the time of extraction, approximately 9-10 kg(20-22 lbs) of placental cotyledons were homogenized with a Polytron PT-10 (Brinkmann) homogenizer at maximum speed for 30-60 seconds in 0.05M glycine-hydroxide buffer, adjusted to pH 9.5 with 1 N ammonium hydroxide, using a ratio of buffer to tissue of 5:1 (v/w). The homogenate was stirred overnight and then centrifuged at 20,000 x g for 20 min. The pellet was discarded.

2. Acidification:

To the supernatant was added slowly glacial acetic acid to a final pH of 6.5 with constant stirring. After allowing the precipitate to settle overnight, the mixture was centrifuged at 20,000 x g for 20 min. , the precipitate was discarded . To the supernatant was added slowly 2 N ammonium hydroxide to achieve a final pH of 9.0.

3. Anion Exchange Chromatography:

The pH 9.0 solution was diluted with an equal volume of distilled water and was applied to a column (15 x 50 cm) of diethylaminoethyl cellulose (Whatman DE-32) previously equilibrated with 0.01 M glycine-hydroxide buffer, pH 9.0. After the column was washed with 3 to 4 bed volume of starting

buffer, the eluates were pooled. The materials bound to the column were discarded.

4. Cation Exchange Chromatography:

The pooled eluate was acidified slowly with glacial acetic acid to a final pH of 6.0 . After stirring the solution overnight, the solution was centrifuged at 780 x g for 20 min. The precipitate was discarded.

The supernatant was applied to a column (15 x 50 cm) of carboxymethyl cellulose (Whatman CM-32) which was also equilibrated with 0.01 M ammonium acetate buffer, pH 5.5. After washing the column with 20 liters of starting buffer, 0.01 M ammonium acetate, pH 5.5, the column was eluted with a stepwise NaCl gradient (0.05, 0.1, 0.2, and 0.5M). the fractions containing oPL were pooled .

5. Carboxylmethyl- Sephadex column chromatography:

The pooled fractions were further diluted with 3 equal volumes of distilled water, and applied to a column of carboxymethyl-sephadex (CM-Sephadex C-50,Pharmacia; 6 x 15 cm) which was equilibrated with 0.01 M sodium acetate, pH 6.0. After the column was washed with 5 bed volumes of 0.01M sodium acetate buffer, pH 6.0 containing 0.05M NaCl, a linear NaCl gradient was begun using 0.01M sodium acetate, pH 6.0 containing 0.05M sodium chloride as the initial buffer and 0.01M sodium acetate, pH 6.0 containing 0.5M NaCl as the limiting buffer. The fractions containing oPL were pooled and concentrated to a small volume by ultrafiltration by using an Amicon membrane UM10.

6. Sephadex G-100 gel filtration:

The concentrated material was applied to a Sephadex G-100 (Pharmacia, Uppsal, Sweden) column (4.2 x 104 cm) which was equilibrated with 0.01M glycine-hydroxide buffer, pH 10.0. Fractions containing oPL were collected and pooled.

7. Diethylaminoethyl-Sephadex column chromatography:

The pooled fractions were applied directly onto a column (1 x 20 cm) of diethylaminoethyl- sephadex (DE-Sephadex A-25, Pharmacia) which was equilibrated with 0.01M glycine-hydroxide buffer, pH 10.0. After the column was washed with 10 bed volumes of eluting buffer, 0.01M glycine-hydroxide buffer, pH 10.0., a linear NaCl gradient was begun using 0.2M sodium chloride as the final salt concentration. The fractions containing oPL were collected, pooled, and concentrated into a small volume.

8. Gel filtration:

Finally, the concentrated material was applied to a column (1.4 x 9 4 cm) of Sephadex G-50 (Superfine, Pharmacia) which was equilibrated with 0.05 M ammonium bicarbonate, pH 8.2. The active fractions were pooled, concentrated to a volume of 2 to 3 ml, and then lyophilized.

Characterization Procedures

1. Chemical

(a) Analytical gel electrophoresis:

Polyacrylamide gel electrophoresis was carried out as described by Davis(1964) and Reisfeld et al (1962) with slight modification. In alkaline gel, 7.2% acrylamide and pH 9.5 were used, whereas under acidic condition, 9.0% acrylamide and pH 4.3-4.5 were used. In both conditions, duplicate samples were run . One gel was stained with dye while the other was cut serially and the individual segments were eluted in 1 ml of 0.1 M Tris-HCl, pH 7.6 containing 0.1% BSA at 4 C for 24 hours with shaking. The eluants subsequently were analyzed by the radioreceptorassay for growth hormone.

The staining was performed by placing the gel in 1% Amido Black dye (in 7% acetic acid) for 1 or 2 hours. The stained gel was removed from the staining solution and placed in a test tube with 7% acetic acid to destain until distinct bands were seen.

For the alkaline gel, bromophenol blue was used as a tracking dye, whereas for the acidic gel, Basic Fuchsin dye was used.

(b) Analytical gel isoelectric-focusing electrophoresis:

Analytical thin layer polyacrylamide gel isoelectric-focusing was performed using a LKB 2117 multiphor apparatus. Focusing was achieved using a pH range 3.5 to 9.5 with a 6% (w/v) concentration of acrylamide and a cross-linking of 2.5%.

The thin-layer polyacrylamide gels containing Ampholine carrier ampholytes were supplied by the LKB-Produkter AB, S-161 25 Broma, Sweden. Duplicate samples were run at the same time, one gel was placed in a fixing solution containing methanol(30%), sulphosalicylic acid (3.26%), and trichloro-acetic acid (TCA, 10.86%). Then the gel was stained with 0.115% Coomassie Brillant Blue R250 in a solution containing 25% ethanol, 8% acetic acid. The other gel was cut serially (0.5 cm in width) and the individual segments were eluted in 1 to 2 ml of 0.1 M Tris-HCl, pH 7.6 containing 0.1% BSA (w/v) for 24 hours at 4 C. Subsequently the eluants were analyzed by RRA-GH.

The staining procedures were carried out at 22 C for 2-3 hours, and then destained for 36-48 hours in a solution containing water, ethanol, and acetic acid in a ratio of 8:3:1 respectively.

One additional blank gel run at the same time was divided serially (0.5 cm in width) and the individual segments were eluted with distilled water for 24 hours at 4 C with shaking. The pH of the eluant was determined by pH electrode (Fisher, Acumet, Model 420).

(c) Analytical sodium dodecyl sulfate polyacrylamide gel (slab) electrophoresis

Polyacrylamide gel electrophoresis containing sodium dodecyl sulfate was carried out as described by Weber and Osborn (1969) with slight modification. For the resolving gel, a 12% acrylamide was used, whereas for the stacking gel, a 3% acrylamide was used. Electrophoresis was allowed to proceed at a constant current of 20 milli-amperes. The samples were boiled at 90 C for 1 minute with 2% sodium dodecyl sulfate, 2% glycerol, and small amount of bromophenol blue before being applied to the top of the stacking gel. For each sample, 10 ug of protein was used.

The staining was performed by placing the gel in 50% TCA containing 0.25% Coomassie Brillant Blue R250 for 2-3 hours. Then the stained gel was removed from the staining solution and destained in destaining solution containing 10% methanol and 5% acetic acid.

(d) Molecular weight determination:

The molecular weight of oPL was estimated by a comparision of the mobility of purified oPL with the mobility of proteins of known molecular weight in 12% polyacrylamide gels containing Sodium dodecyl sulfate as described above.

3. Receptor assays:

Displacement curves of oPL in the radioreceptorassay for prolactin (RRA-PRL) and for growth hormone (RRA-GH) were determined using rabbit mammary gland and liver respectively.

The purified oPL preparation was accurately weighed and dissolved in 0.05M ammonium bicarbonate solution. Serial dilutions of oPL were made in 0.025M Tris-HCl, pH7.6 containing 0.1% BSA. The radioreceptorassay for prolactin (RRA-PRL) was performed according to the method of Shiu et al(1973) as described previously. The radioreceptorassay for growth hormone was performed according to the method of Tsushima et al(1973) as described previously except that [^{125}I]-iodo oGH and oGH (NIH 0-986L, 2.0 IU/mg) were used as tracer and standard respectively.

[^{125}I]-iodo oGH was prepared by the lactoperoxidase enzymatic method described by Thorell and Johannson(1971) with slight modification. During iodination, the pH of 0.05M phosphate buffer added in the reaction mixture is pH 7.0, 5 ug of lactoperoxidase, 10 ul of 30% hydrogen peroxide (1:1,500 dilution), and reaction period of 20 min. were used. The percentage of radioreactivity incorporated was 55-60, and the specific activity was 100-130uCi/ug of protein.

B : METHOD OF DETECTION AND CHARACTERIZATION OF THE RECEPTOR BINDING SITES FOR OVINE PLACENTAL LACTOGEN IN THE SHEEP

MATERIALS AND METHODS

Tissue Receptor Preparation

Sheep tissue were obtained within 5 min of the death of the animals and were immediately frozen at -20 C until required. All tissues were processed within 6 months. At the time of preparation of 100,000 x g microsomal fractions, tissues were thawed and homogenized in ice cold 0.3M sucrose in a polytron PT-10(Brinkmann Instruments, Inc., Westbury, N.Y.) homogenizer set at maximum speed for 30-60 sec, except for the adipose tissues which were homogenized with 0.3M sucrose at 22C. The ratio of tissue to sucrose was 1:5 (wt/vol). After homogenization, centrifugation was carried out as described previously (Shiu et al,1973). Initially, the homogenate was centrifuged at 3,000 rpm for 30 min. The supernatnant was separated from the pellet(780 x g fraction) and subjected to a second period of centrifugation at 11,500 rpm for 20 min. Again the supernatant was separated from the pellet(15,000 x g fraction) and was subjected to final centrifugation at 45,000 rpm for 90 min. The pellet obtained at the final stage was called the 100,000 x g microsomal pellet. The pellet was suspended in 0.025M Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and stored at -20C until use.

Iodination of oPL

Ovine [^{125}I]iodo-PL was prepared by the lactoperoxidase method of Thorell and Johansson(1971) with slight modifications. For iodination, 0.05M phosphate buffer(25 ul) added to the reaction mixture was adjusted to pH 4.2 instead of pH 7.4 and 5 ug oPL, 5 ug lactoperoxidase, 10 ul of 30% hydrogen peroxide (1:1,500 dilution), and a reaction period of 10 min were used. Unreacted iodide and damaged hormone were separated from intact ovine [^{125}I]iodo-PL by gel filtration on a Sephadex G-100 column (1.5 x 50 cm) using 0.025M Tris-HCl, pH 7.6, as eluting buffer. The percentage of radioactivity incorporated was 50-60%, and the specific activity was 100 to 120 uCi/ug protein. Using this procedure, 8 to 10 consecutive iodinations yielded a satisfactory tracer.

Tests of Integrity of Ovine [^{125}I]iodo-PL

Fractions eluted from the Sephadex G-100 column were tested for specific binding in the rabbit liver receptor assay(Tsushima et al,1973). Only fractions which exhibited specific binding greater than 15%/250 ug protein were considered satisfactory for subsequent use in assays, because satisfactory [^{125}I]iodo-hGH preparations generally bind to this extent.

All hormone preparations and ovine [^{125}I]iodo-PL were diluted in 0.025M Tris-HCl, pH 7.6, containing 0.1% bovine serum albumin(BSA) and 10 mM $MgCl_2$.

Assay Procedures

Specific binding studies were conducted with 1000 ± 50 ug protein, concentration being determined by the method of Lowry et al (1951). To each assay tube was added 0.2 ml of 0.025M Tris-HCl buffer, pH 7.6 containing 0.1%BSA and 10 mM $MgCl_2$, 0.1 ml 100,000 x g pellet suspension, 0.1 ml ovine [^{125}I]iodo-PL (80 - 100,000 cpm) with or without 1 ug of oPL in 0.1 ml of buffer, for a total volume of 0.5 ml. The samples were incubated at 4 C for 24 hours. The reaction was terminated by the addition of 3 ml ice-cold 0.025 M Tris-HCl buffer containing 0.1% BSA and 10 mM $MgCl_2$. Bound and free hormone were separated by centrifugation at 780 x g for 20 min at 4 C. The supernatant(free hormone) was decanted by inverting the tubes and allowing them to drain for 30 min, and the membrane bound ovine [^{125}I]iodo-PL in the precipitate was determined by counting the radioactivity in an LKB autogamma counter.

The percentage of specific binding of ovine [^{125}I]-iodo PL to the tissue was calculated from the formula:

Specific binding = [cpm bound to the tissue in the absence of unlabelled oPL - cpm bound in the presence of a large excess (1 ug) of unlabeled oPL] X 100/ total cpm of ovine [^{125}I]iodo-PL added

In the radioreceptorassay assay for oPL using various ovine tissues (100,000 X g pellet), the assay procedures were identical with the above (specific binding studies) except that a known amount (various concentrations) of unlabeled hormone or crude tissue extract was added to the reaction

tube instead of a single dose (1 ug) in order to obtain a standard curve.

Radioreceptorassay for oPRL using ovine tissue receptors

The assay procedures were identical with the radioreceptor assay for oPL except that ovine [^{125}I]iodo-PRL, oPRL (NIH-P-S-10, 26 IU/mg), and pregnant ewe uterus (90-135 days of gestation, 100,000 x g pellets) were used as tracer, hormone standard, and receptors, respectively.

C : METHOD OF DEVELOPMENT OF A SPECIFIC AND SENSITIVE RADIOIMMUNOASSAY FOR OVINE PLACENTAL LACTOGEN

MATERIALS AND METHODS

Immunization Procedures

Eight New Zealand white rabbits(2-3 kg) were injected subcutaneously (sc) once a week for 3 weeks with 200 ug of oPL dissolved in 0.5 ml 0.1M ammonium bicarbonate and emusified with an equal volume of complete Freund's adjuvant. Thereafter, the animals were injected sc at 3 week intervals with 50 ug oPL/animal. The animals were bled 1 week after the third injection and the serum was tested for antibodies to oPL.

Iodination of oPL

Ovine [^{125}I]iodo-PL was prepared by a slight modification of the lactoperoxidase method of Thorell and Johansson (1971) as described in the previous section (Section V).

Tests for Integrity of Ovine [^{125}I]iodo-PL

The ovine [^{125}]iodo-PL was tested for specific binding in the rabbit liver receptor assay(Tsushima et al,1973)as described in the previous section(Section V).

Radioimmunoassay Procedures

The double-antibody radioimmunoassay (RIA) procedure was similar to that described by Beck et al (1967).

All hormone preparations, pituitary and placental extracts, serum, and oPL tracer were diluted in 0.01M phosphate-buffered saline(PBS), pH 7.6, containing 2.5% BSA, and a 0.1 ml sample (or 0.2 ml sample) was taken for assay. Approximately 30,000 to 35,000 cpm ovine [^{125}I]iodo-PL in 0.1 ml buffer, 0.1 ml diluted antiserum to oPL (1/50,000 dilution), 0.1 ml oPL standard or assay samples, and 0.5ml PBS, pH 7.4, containing 2.5% of BSA were added to glass tubes (12 X 75 cm). After a 72-h incubation at 4 C, 0.1 ml sheep anti-rabbit gamma-globulin serum (1/35 dilution) and 0.1 ml normal rabbit serum (1/350 dilution) were added. After a further 24-h incubation period in the cold, the precipitates formed were centrifuged at 780 X g for 30 min, and the supernatant was decanted. The radioactivity of the precipitates was then counted in a LKB gamma-counter (model 8000).

Pituitary and Placental Extracts

Pituitary and placental tissues from various species were homogenized with 0.1 M NH_4HCO_3, pH 8.4 at 4C with a tissue to buffer ratio of 1:5 (wt/vol), stirred overnight, and centrifuged at 15,000 x g for 20 min; the pellet was discarded and the supernate was stored at -20 C until assay.

D. STUDIES ON THE SECRETION OF OVINE PLACENTAL LACTOGEN DURING PREGNANCY

(1) Maternal and fetal concentration of ovine placental lactogen measured by radioimmunoassay

Materials and Methods

Animals and Blood Samples:

The majority of the ewes were of the Suffolk breed and the remainder were a mixed crossbreed. Breeding rams were fitted with a marking harness and the time of estrus and mating was determined by visual examination of the ewes twice per day for signs of marking. The day of first marking was designated as day 0 of pregnancy. Peripheral blood samples (20ml) were taken from 11 ewes between 0800 and 0900 h at regular intervals throughout pregnancy without anesthesia for longitudinal studies. For acute or cross-sectional studies, samples of maternal and fetal fluids were obtained as follows. Ewes at different stages of pregnancy were anesthesized with Halothane and samples of maternal peripheral jugular and uterine venous blood were obtained while under anesthesia, the ewes were then sacrificed and samples of amniotic, allantoic, and fetal blood were taken. The allantoic fluid was collected in a syringe by aspiration after the insertion of an 18 gauge needle through the choio-allantoic membrane. The chorio-allantoic membrane was then ruptured to let the residual allantoic fluid drain off before the

aspiration procedure was repeated for the collection of the amniotic fluid. At the early stages of pregnancy, fetal cord blood was obtained from the umbilical cord(vein), whereas during the later stages, fetal blood was obtained by heart puncture.

Blood samples were collected, either with or without added anticoagulant. Upon withdrawl, the blood was immediately chilled in ice, centrifuged at 4 C within 2 h, and 3 ml aliquots of plasma or serum were frozen and stored in stoppered glass vials at -20 C. For oPL determination, a 3 ml aliquot was thawed at room temperature. samples stored for as long as 2 years showed no apparent loss of oPL content.

(2) Ovine placental lactogen concentration in the maternal and fetal cotyledons at various days of gestation

Placentomes were collected from ewes sacrificed at various days of gestation. The maternal caruncles were separated from the fetal membrane (fetal cotyledons plus fetal intercotyledonary membranes) until day 40, but , subsequently, no separation was carried out between maternal caruncles and fetal cotyledons. As soon as the tissues were collected, they were immediately frozen over solid carbon dioxide, sealed in plastic bags, and stored at -20 C until use.

For determination of oPL concentration in the tissues, the placental tissues were weighed, homogenized with 0.1M ammonium bicarbonate buffer, pH 8.2 at 4 C with a tissue to buffer ratio of 1:5

(wt/vol). After the homogenate was extracted overnight, the homogenate was centrifuged at 15,000 x g for 20 min; the pellet was discarded and the supernate was assayed immediately by RIA-oPL or was stored at -20 C until assay.

(3) Determination of half-time disappearance rate of oPL in the ewes

Two methods were employed to determine the disappearance rate of oPL from circulation. In the first approach the uterus and placenta of the pregnant ewes were removed, and the concentration of oPL measured in the circulation at various times before and after surgery. The second approach involved measurement of the disappearance of radioactively labeled oPL from samples taken at various times after injection of radioactive oPL.

(i) Surgical method:

Three pregnant ewes at 90 days of gestation were anesthetized with Halothane, and samples of maternal peripheral jugular venous blood were obtained before surgical removal of the entire uterus. Blood samples also were collected at different time intervals after surgical removal of the entire uterus up to 170 to 230 min later. After the blood samples were collected, they were immediately centrifuged at 3,000 rpm for 30 min at 4C and the plasma were obtained and stored at -20 C until assay for oPL concentration.

(ii) Method by injection of radioactive oPL:

Two pregnant ewes (35 days of gestation) were anesthetized with Halothane and the jugular blood samples were obtained before

injection of ^{125}I-oPL. ^{125}I-oPL (approximately 2.5 x 10^7cpm/10 ml) was injected intravenously into the left jugular vein and heparinized blood samples (5 ml) were collected at frequent intervals from the right jugular vein. Blood loss from the circulation was compensated for by the addition of 5 ml heparinized saline into the jugular vein before each sampling. After collection, the blood samples were immediately centrifuged at 3,000 rpm for 30 min at 4C and the serum was maintained at 4 C until use.

Serum sample (one ml) was counted in the gamma counter. Then two ml of 10% (w/v) ice-cold trichloroacetic acid(TCA) was added and the mixture was vigorously shaken. The precipitate obtained after incubation at 4 C for 1 h was collected by centrifugation at 3,000 rpm for 30 min at 4 C, washed twice with ice-cold TCA and counted.

For the determination of half-time disappearance rate of ^{125}I-oPL, the blood samples were taken as described above at different intervals and the plot of the logarithm of the radioactivity present in both the blood and the TCA-insoluble material as function of time was obtained. Two half-times were calculated: $t_{\frac{1}{2}}(S)$, which represents the half-time calculated from the regression line obtained by subtracting the extrapolated values of the line of best fit over 10 to 25 min from the values corresponding to the line drawn through the points up to the first 10 min. The half-time, $t_{\frac{1}{2}}(L)$ was calculated directly from the formula: 0.693/slope of $t_{\frac{1}{2}}(L)$. Similarly for $t_{\frac{1}{2}}(S)$= 0.693/slope of $t_{\frac{1}{2}}(S)$.

(4) Tissue distribution of radioactivity after intravenous administration of ^{125}I-oPL

^{125}I-OPL (approximately 2.5 x 10^7cpm / 10 ml) was injected as described above in (3). After 240 minutes of administration of ^{125}I-oPL, a blood sample was collected and the animals were killed immediately by giving a large amount of potassium chloride intravenously. Tissues were quickly removed, blotted dry, weighed and the radioactivity present was determined in a gamma counter. The results were expressed as counts/min/gm tissue divided by the counts/min/ml of whole blood(organ/blood ratio, O/B).

(5) Studies on the relationship among ovine pituitary growth hormone (oGH), prolactin(oPRL), placental lactogen(oPL), and progesterone in the ewes during pregnancy

In order to elucidate the functional relationships among oGH, oPRL, oPL, and progesterone in the ewes during pregnancy, these hormones were measured simultaneously using homologous radioimmunoassay for each hormone except progesterone. Progesterone concentration was kindly determined by Dr. H.A. Robertson using a competitive-binding assay. The procedures were performed according to the method described by Robertson et al (1971) using dog plasma corticosterone-binding globulin(CBG) as binding protein.

The RIAs for oGH and for oPRL were developed using the same method as for oPL,except that oPRL(NIH-P-S-10) and oGH(NIH,o-984) were used for raising the antibodies in the rabbits.

E. STUDIES ON THE BIOSYNTHESIS OF OVINE PLACENTAL LACTOGEN BY THE CHORIONIC MEMBRANES, in vitro

(1) Culture method

Ovine chorionic membranes were obtained immediately after surgery of pregnant ewes (26, 35, 55 days of gestation). Chorionic membranes from several ewes of same gestational period were pooled and cut into fragments, rinsed with ice-cold Hank's balanced salt solution. Approximately 100 mg fragments were placed into a 20 ml glass flask and incubated for 30 min in 10 ml of Dulbecco's Modified Eagle Medium(DMEM) obtained from Gibco, New York. Each flask contained 100 U of Penicillin-Streptomycin solution. After 30 min preincubation, the medium was replaced with 10 ml of Dulbecco's Modified Eagle Medium (without leucine) containing 50 uCi/ml of ^{3}H-leucine (specific activity, 70-100 Ci/mmol, New England Nuclear Corporation). The incubation was carried out at 37 C in an atmosphere of 95% O_2 and 5% CO_2. After 24 hours of incubation, the medium and the tissues were frozen at -20 C immediately until use.

Flasks containing chorionic membranes were set up at least in duplicate and analyzed separately.

(2) Processing of incubation media

The frozen 24 h incubation medium and tissue fragments were thawed and centrifuged at 3,000 x g. The supernatant was further centrifuged at 50,000 x g for 30 min and the clear supernatant was frozen at -20 C until use.

(3) Radioimmunoassay for oPL content

The oPL content in the media was determined by the double antibody radioimmunoassay as described previously.

(4) Determination of the synthesis and secretion of oPL

The newly synthesized oPL was determined by measuring the incorporation of ^{3}H-leucine into oPL molecule. The quantities of ^{3}H-oPL in the medium was determined by immunoprecipitation using specific antiserum to oPL prepared in rabbits as described previously.

(i) Method of determination of the equivalence zone for oPL antiserum.

The equivalence zone for maximal precipitation of radioactive oPL was determined as follows. 0.1 ml of cold oPL in various concentration (10 to 100,000 ng/ml) was added to test tubes containing 300 ul of phosphate buffered saline(PBS), pH 7.6, 25 ul oPL antiserum, and 20,000 cpm of ^{125}I-oPL. The final volume was approximately 525 ul. The mixture was then incubated for 1 h at 37 C, and then for an additional 16 h at 4 C. After overnight incubation (16 h), 2 ml of PBS were added into the reaction mixture, and then centrifuged at 3,000 x g for 30 min. The pellets were washed and re-centrifuged twice

in the same buffer. Then the pellets were counted in the gamma counter. In this study, the maximal precipitation of ^{125}I-oPL, in the range of 80-90%, occurred when 500 to 2,000 ng carrier oPL was added to incubation tubes containing 25 ul of antiserum to oPL.

(ii) Immunoprecipitation of ^{3}H-oPL in the media

Consequently, in order to assure maximal precipitation of ^{3}H-oPL in the media, sufficient carrier oPL (1,000 ng) was added to each incubation tube containing 25 ul antiserum, 100 ul of medium, and 300 ul of PBS. After the mixture was incubated for 1 hour at 37 C and an additional 16 h at 4 C, 2 ml of PBS containing 5 mM leucine were added to stop the reaction, then the reaction tubes were centrifuged at 3,000 x g for 30 min. The pellets were washed and re-centrifuged twice in the same buffer and dissolved in 0.5 ml of 0.1M NaOH. Each sample was added to 10 ml of Aquasol II (New England Nuclear, Boston) and the tritium was counted in a Beckman liquid Scintillation spectrophotometer.

In order to account for nonspecific sticking of labeled proteins to the incubation tubes, identical quantities of medium were assayed as described above except that non-immune rabbit serum was used in place of specific antiserum and the counts in these tubes were substracted from those in tubes containing specific antiserum.

(iii)Determination of the total ^{3}H-proteins in the media

To determine the total quantities of ^{3}H-proteins in the media, 2 ml of 10% TCA containing 5 mM leucine were added to 100 ul of medium. The precipitate was obtained by centrifugation at 3,000 x g

for 30 min. Then the precipitate was resuspended in 2 ml of 5% TCA, and re-centrifuged. Finally, the precipitate was dissolved in 0.5 ml of 0.5N NaOH, re-precipitated with 5% TCA, recentrifuged, and the precipitate was dissolved in 0.1 M NaOH and counted in Aquasol II as described above.

(5) Chromatography of the incubation medium

3 ml of incubation medium was chromatographed on a Sephadex G-100 column (45 X 2 cm) which was equilibrated and eluted with 0.01 M PBS containing 5mM leucine. Fractions of 3 ml were collected. The effluent fractions were assayed for total oPL by radioimmunoassay, ^{3}H-oPL by immunoprecipitation, ^{3}H-protein by TCA precipitation, and total protein concentration by measuring the absorbance at 278 nm.

F. STUDIES ON THE BIOLOGICAL EFFECTS OF OVINE PLACENTAL LACTOGEN

(1) Assay of growth promoting activity in hypophysectomized rats-

The tibia test for growth promoting activity was performed by the Endocrine Laboratories of Madison, Inc., Wisconsin 53713, U.S.A. The experimental procedures for the assay are the following:

Immature Sprague -Dawley rats were hypophysectomized at 30 days of age and held for 12 days under closely regulated conditions of temperature and humidity. At the end of this 12 day acclimatization period, animals exhibiting inappropriate weight gain were discarded. Partially purified oPL (GH potency (1.0 - 1.3 IU/mg) estimated by RRA-GH using hGH as standard) or bovine growth hormone (0.9 IU/mg) were prepared for injection using equal volumes of chilled saline (0.9% NaCl) and 0.05 M ammonium bicarbonate, pH 7.8. If necessary, diluted NaOH (0.01N) was added to dissolve the hormone preparations but the final pH was never greater than 9.5. The solutions containing saline, oPL or bGH were administered by intra-peritoneal injection once daily for 4 days. The animals were sacrificed by decapitation on the day following the last day of injection. Both tibae of each rat were dissected, split longitudinally and prepared for measurement of epiphyseal width. Weights of adrenals and ovaries were recorded. The potency estimates of oPL were calculated statistically using bGH (0.9 IU.mg) as standard.

(2) Assay of luteotropic effect of ovine placental lactogen in the pseudopregnant rat

The functional relationship between ovine placental lactogen and corpus luteum function was assessed by the ability of ovine placental lactogen to maintain or to prevent the loss of LH-receptors in the corpora lutea of the pseudopregnant rat after prostaglandin F_{2a} treatment. It has been reported that administration of prolactin could block the effects of prostaglandin F_{2a}(PGF_{2a}) on the LH-receptors in the corpora lutea of the pseudopregnant rat (Grinwich et al,1975).

(i) Animals:

Immature female rats (Sprague-Dawley) were purchased from Canadian Breeding Farm and Laboratory Ltd, Montreal, Canada. At 24 days of age they were treated with 50 IU of pregnant mare serum gonadotropin (PMSG) obtained from Ayerst Laboratory, Montreal, Que. Sixty-four hours later they were given a second injection of 25 IU of human chorionic gonadotropin (A.P.L., Ayerst, Montreal, Que.). Then the animals were randomly assigned into groups of five. Five days following the human chorionic gonadotropin injection, the animals received either hormone treatment or saline. The type of hormone preparations and the time schedule of adminstration are shown in the following tables (Experiment # I and II).

EXPERIMENT # 1

GROUP NUMBER	(5 days after HCG) 0090	1700	(6 days after HCG) 0090	1400	1700	(7 days after HCG) 0090
#1	saline	saline	saline	saline	saline	sacrificed
#2	oPRL*	oPRL	oPRL	oPRL	oPRL	sacrificed
#3	oPRL	oPRL	oPRL	PGF_{2a}****	oPRL	sacrificed
#4	oPL**	oPL	oPL	oPL	oPL	sacrificed
#5	oPL	oPL	oPL	PGF_{2a}	oPL	sacrificed
#6	hPL***	hPL	hPL	hPL	hPL	sacrificed
#7	hPL	hPL	hPL	PGF_{2a}	hPL	sacrificed
#8	saline	saline	saline	PGF_{2a}	saline	sacrificed

*oPRL (NIH-P-S-10, 26 IU/mg),150 ug dissolved in 0.2ml of saline per dose

**oPL, partial purified. Potency equivalent to 150 ug of oPRL standard as assayed in the RRA-PRL were given per dose(150 ug of oPL)

***hPL, purified in our laboratory. Potency equivalent to 150 ug of oPRL standard as assayed in the PRL-RRA were given per dose (300 ug of hPL)

****PGF_{2a}, they were obtained from Sigma,USA. They were given at 200 ug per dose per animal.

EXPERIMENT # II

GROUP NUMBER	(5 days after HCG) 0090	1700	(6 days after HCG) 0090	1400	1700	(7 days after HCG) 0090
#1	saline	saline	saline	saline	saline	sacrificed
#2	oPRL	oPRL	OPRL	PGF_{2a}	oPRL	sacrificed
#3	oPRL	oPRL	oPRL	CB154	oPRL	sacrificed
#4	oPL	oPL	oPL	PGF_{2a}	oPL	sacrificed
#5	oPL	oPL	oPL	CB154	oPL	sacrificed
#6	hPL	hPL	hPL	PGF_{2a}	hPL	sacrificed
#7	hPL	hPL	hPL	CB154	hPL	sacrificed
#8	saline	saline	saline	CB154	saline	sacrificed
#9	saline	saline	saline	PGF_{2a}	saline	sacrificed
#10	saline	saline	saline	CB154&PGF_{2a}	saline	sacrificed

N.B. The amount of hormone preparations used were similar to the doses used in Experiment #1.

200 ug of PGF_{2a} was used per dose or 200 ug of CB154 per dose was given per animal.

CB154 were obtained from Sandoz, Switzerland.

When the animals were killed, bloods were collected and centrifuged after one hour in the cold room to obtain the serum. The serum samples were immediately frozen at -20 C until use. At the same time, the entire luteinized ovaries were removed, trimmed, and immediately frozen at -20 C until further analysis.

(ii) Progesterone assay-

Serum progesterone was quantitated by radioimmunoassay utilizing antisera developed by Merck Institute, Rahway , New Jersey, USA (a gift from Dr. D. Grinwich). The assays were conducted according to the method described by Orczk et al (1974) with slight modifications. The procedures were described as follows:

a- Extraction:

0.1 ml of rat serum was extracted with 5 vol of petroleum ether(Fisher, analytical grade). The extracts were dried under nitrogen. Then appropriate volume of 0.01M PBS containing 0.01% gelatin were added.

In order to determine the percentage of progesterone that has been extracted, 10,000 cpm of ^{3}H-progesterone was added into each sample of sera, and extracted as described above to determine the efficiency of extraction.

b- Assay:

To each assay tube was added 0.1 ml of PBS containing various concentrations of progesterone or 0.1 ml of unknown sample with appropriate dilution, 0.1 ml of ^{3}H-progesterone (15-20,000 cpm), and 0.1 ml of antibodies

to progesterone (1/300 dilution), for a total of 0.3 ml. The mixtures were incubated at 4 C for overnight. Then the reaction was terminated by the addition of 0.5 ml of ice-cold charcoal-dextran solution containing 0.5% charcoal(Norite A, Neutral, Fisher), 0.1% Dextran(T70, Pharmacia). The bound and free hormones were separated by centrifugation at 780 x g for 10 min at 4 C immediately after addition of charcoal-dextran solution. The supernatant was removed with a pasteur pipette and added to a scintillation vial containing 10 ml of Bray's solution(0.4% omnifluor, 5% Naphthalenein 1,4-dioxane), andcounted in aBeckman scintillation counter.

(iii) LH-receptor quantitation:

The methodology for quantitating the LH-receptors was based upon the binding of ^{125}I-hCG to the luteal membrane fractions and displaced by excess hCG(Hichens et al,1974). Basically, the following procedures were employed:

Immediately prior to assay, the ovaries were thawed, weighed and homogenized with 0.025M Tris-HCl,pH 7.6 containing 10 mM $MgCl_2$ in a Polytron PT-10 homogenizer (Brinkmann, USA) at 4C. The ratio of tissue to buffer was 0.01 to 1(w/ vol). After homogenization for 30 seconds with the speed set at dial 5, the homogenates were centrifuged at 780 x g for 10 min. The pellets were resuspended in the same buffer and re-centrifuged twice. Finally, the washed pellets were resuspended in the 0.025M Tris-HCl, pH7.6 containing 10 mM $MgCl_2$. The volume used for final resuspension was the same as the original volume for the first homogenization.

The procedures for setting up the radioreceptor assay for LH are the same as described for radioreceptorassay for oPL except that rat ovarian homogenate, ^{125}I-hCG, and hCG were used as receptors, tracer, and hormone standard respectively. A plot of Bound/Free ratio versus Bound gave a Scatchard plot from which the association constant and binding capacity were determined (Scatchard, 1949).

SECTION V : RESULTS

A. PURIFICATION AND CHARACTERIZATION OF OVINE PLACENTAL LACTOGEN

(I) ASSAY EMPLOYED TO MONITOR THE HORMONAL ACTIVITY OF OVINE PLACENTAL LACTOGEN DURING PURIFICATION

Radioreceptorassay for growth hormone-activity using rabbit liver 100,000 X g pellet

The sensitivity of the RRA-GH employed to monitor the growth hormone-like activity of oPL is about 1 ng(10 ng/ml) without serum added as shown in Figure 4. In the RRA-GH, hGH, oGH inhibit the binding of ^{125}I-hGH to rabbit liver 100,000 x g pellet in a parallel manner, hPL and oPRL inhibit minimally, whereas other hormone preparations such as oLH, oFSH, and oTSH(not shown) do not inhibit the binding of ^{125}I-hGH. Ovine placental extract displace ^{125}I-hGH in a parallel manner as shown in the figure.

(II) PURIFICATION AND CHARACTERIZATION OF OVINE PLACENTAL LACTOGEN

Purification

Extraction of oPL from frozen placental cotyledons

The primary extraction was carried out at an alkaline pH of 9.5 (0.1M NH_4HCO_3) because this appears to be an optimal condition to extract most of the oPL from frozen placental cotyledons as compared with other conditions (M.Sc. Thesis,1975).

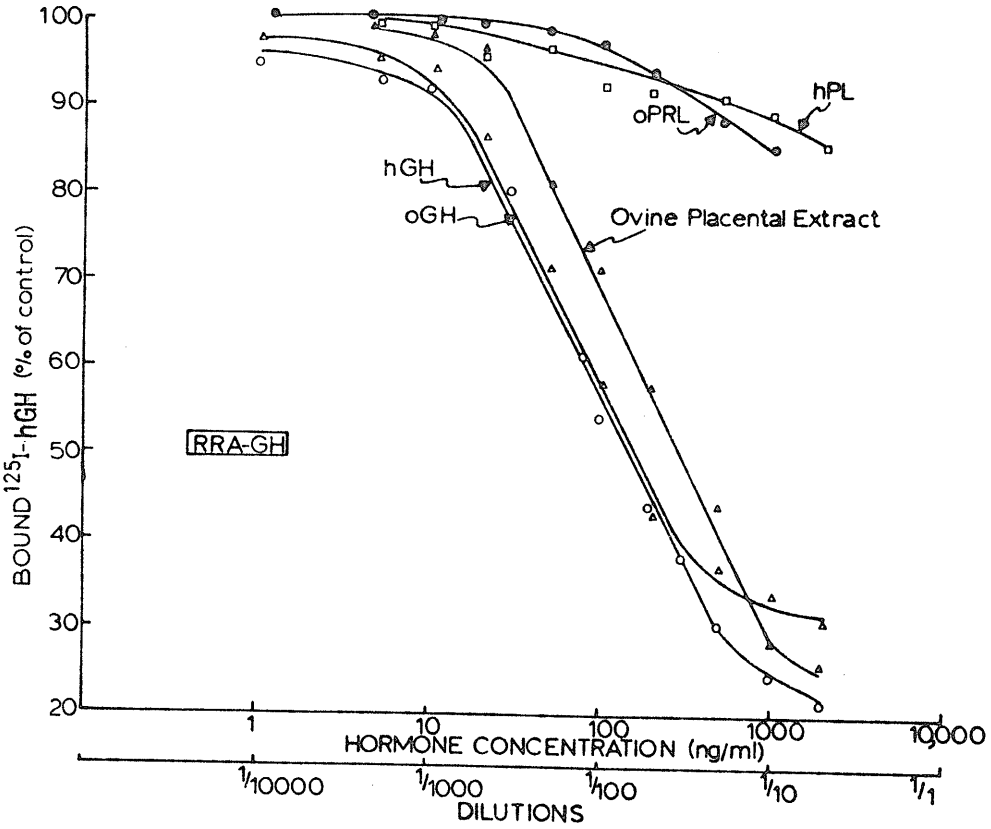

Figure 4. Radioreceptorassay for growth hormone or growth hormone-like activity using 100,000 x g fractions derived from rabbit liver. The sensitivity and specificity of binding of the assay is illustrated (from Tsushima and Friesen,1973)

Fractionation of oPL from crude extract by acidification

Although fractional precipitation by lowering the pH of the crude extract was not the most effective method(M.Sc. Thesis,1975), lowering the pH of the crude extract to 6.5 with glacial acetic acid resulted in precipitation of some mucous substances which interfere with the subsequent anion-exchange chromatography. Using this procedure, I could eliminate about 15% of the original protein content, but retained about 95% of the original growth hormone activity in the extract.

Diethyaminoethyl (DEAE) -cellulose anion exchange chromatography

Before applying the acidified extract to DEAE-ion exchange chromatography, the extract was re-adjusted to a pH of 9.0 with 2N ammonium hydroxide. At this pH, most of the oPL was unabsorbed by the column, whereas a considerable amount of protein was absorbed. With increasing concentrations of NaCl in the presence of 0.01 M glycine-OH, pH 9.0, little additional hormone was eluted as shown in Figure 5. In practice one need not elute with higher salt concentration.

Carboxylmethyl (CM)-cellulose cation exchange chromatography

The fractions containing oPL after DEAE column chromatography were acidified to pH 6.0, centrifuged to remove the precipitates, and applied to a CM-cellulose column for additional purification as shown in Figure 6. When a stepwise salt gradient (0.05M to 0.5M NaCL) was employed to elute the oPL from the column, most of the oPL was eluted by 0.2M NaCL. Only a small amount of oPL was eluted with 0.5 M NaCL. The 0.2M fractions were then further fractionated on the Carboxymethyl-Sephadex column .

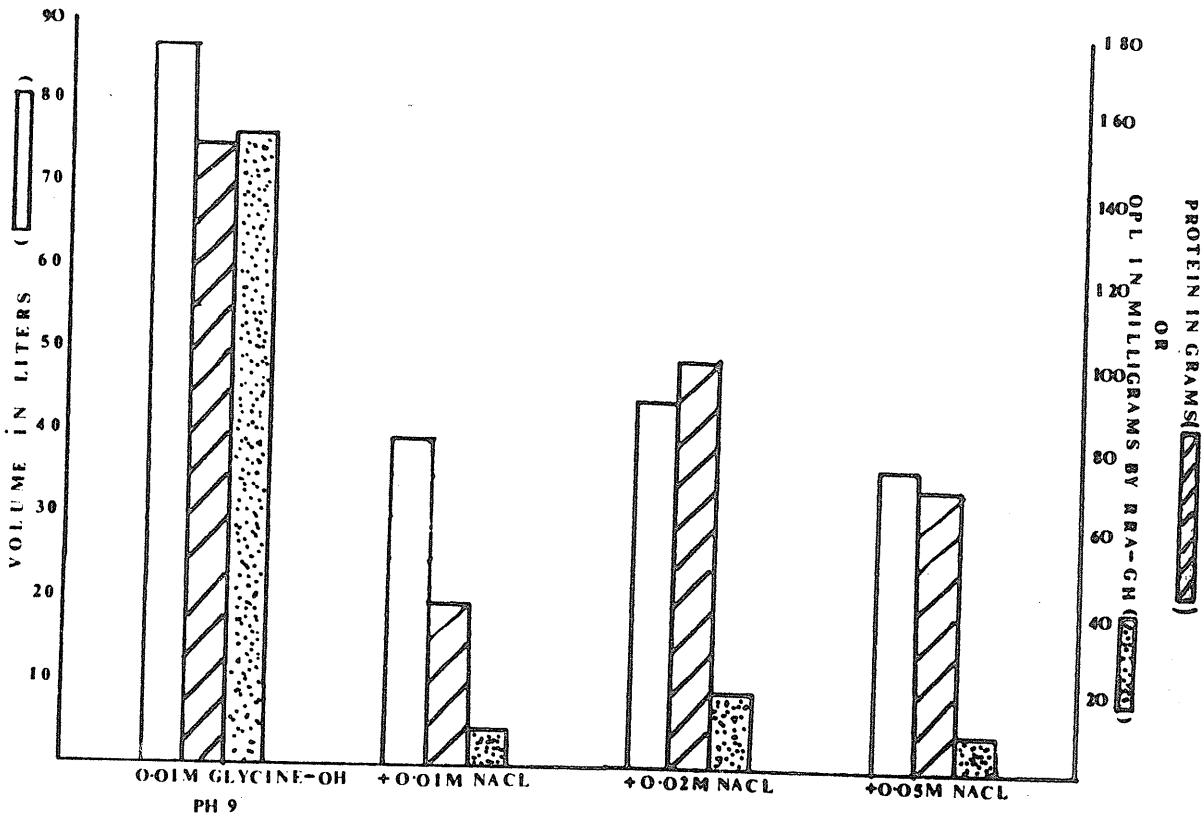

Figure 5. Diethylaminoethyl (DEAE) - Cellulose chromatography of oPL-extract after primary acidification and re-alkalinization.
The DEAE-cellulose (Whatman DE-32) column (15 X 50 cm) was equilibrated with 0.01M glycine-hydroxyl buffer, pH 9.0.

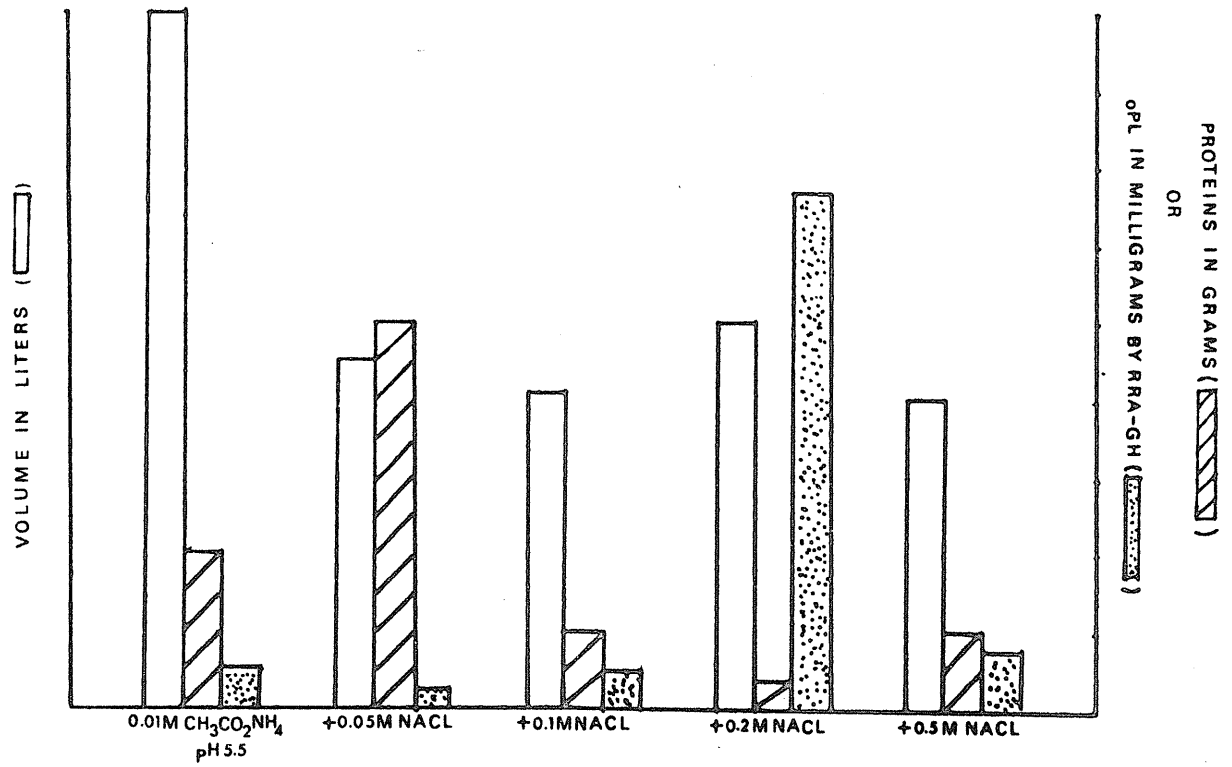

Figure 6. Carboxymethyl(CM)-Cellulose Chromatography of the oPL-rich fractions obtained as shown in Fig. 5. The CM-cellulose column (15 X 50 cm) was equilibrated with 0.01M ammonium acetate buffer, pH 5.5.

Carboxymethyl-Sephadex column chromatography

Figure 7 shows the elution pattern of oPL (0.2M fractions) obtained from CM-cellulose cation exchange column chromatography on a column of Carboxymethyl-Sephadex C-50. A linear gradient (0.05M to 0.5M NaCl) was employed to elute oPL from the column. Most of the oPL was eluted by 0.2M of NaCl in 0.01M ammonium acetate buffer, pH 6.0.

Sephadex G-100 gel filtration

The fractions(# 450-750) containing oPL from Carboxymethyl-Sephadex were pooled, concentrated and further purified by gel filtration on Sephadex G-100 as shown in Figure 8. Most of the oPL eluted at 1.9-2.4 times the void volume.

Diethylaminoethyl-Sephadex column chromatography

The fractions from the Sephadex G-100 gel filtration were pooled and applied to a column of diethylaminoethyl Sephadex A-25 as depicted in Figure 9. When a linear-gradient (0 to 0.05M NaCL) was employed to elute the oPL, most of the oPL was eluted by 0.02M NaCl.

The active fractions were pooled, concentrated, and applied to a column of Sephadex G-50 for final purification.

Gel-filtration

Figure 10 shows the elution pattern of oPL after gel filtration on a column of Sephadex G-50 (Superfine). Most proteins including oPL eluted at 1.5-2.0 times the void volume.

Summary of the results obtained from purification is shown in Table I.

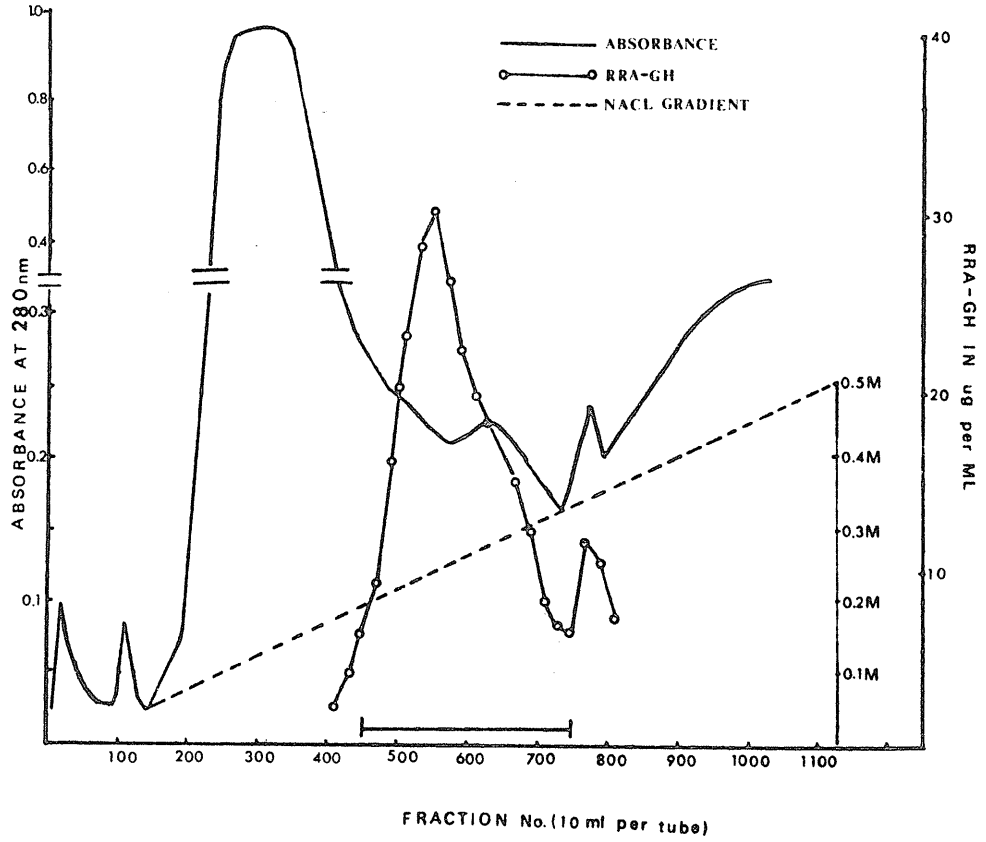

Figure 7. Carboxylmethyl-Sephadex C-50 chromatography of the oPL-rich fractions (0.2 M NaCl fractions) from the CM-cellulose column. The column was pre-equilibrated with 0.01M sodium acetate buffer, pH 6.0. A linear NaCl gradient was employed (0.05 M to 0.5M NaCl) to elute the column.

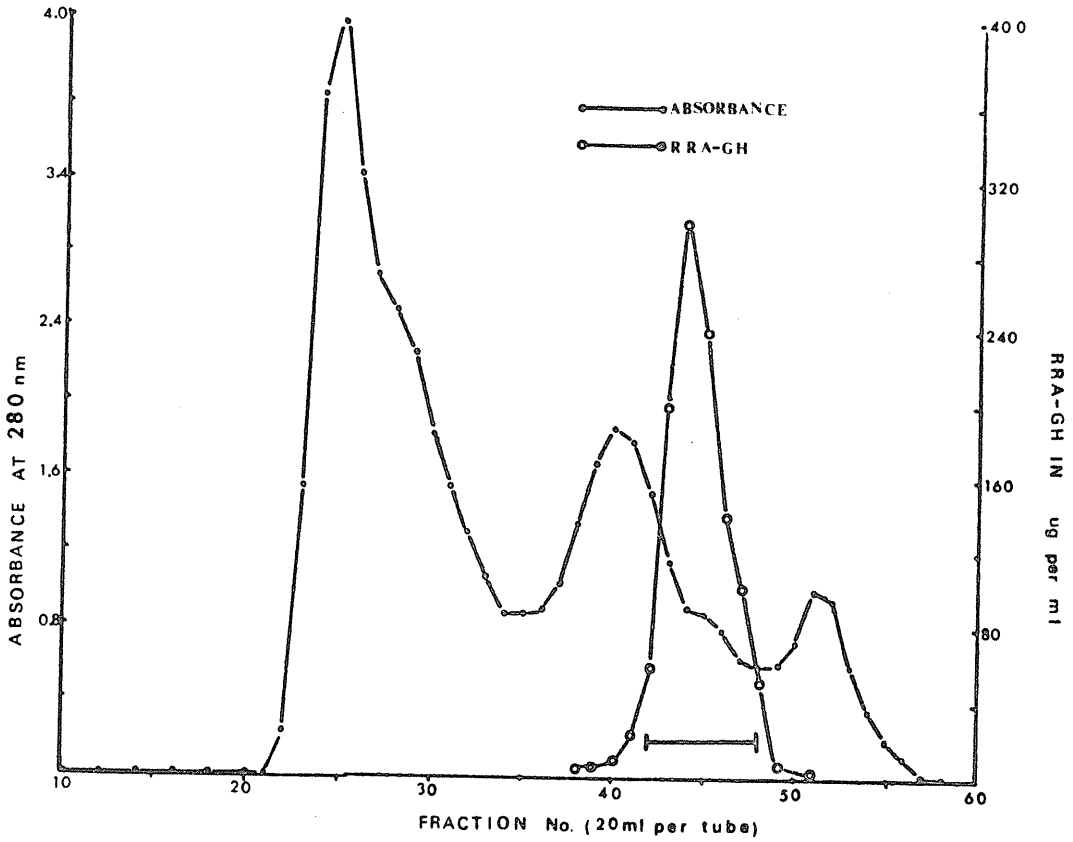

Figure 8. Sephadex G-100 gel filtration of the fractions containing oPL from CM-Sephadex C-50 column (fractions #450-750). The column (4.2 X 104 cm) was pre-equilibrated with 0.01M Glycine-hydroxide buffer, pH 10.0.

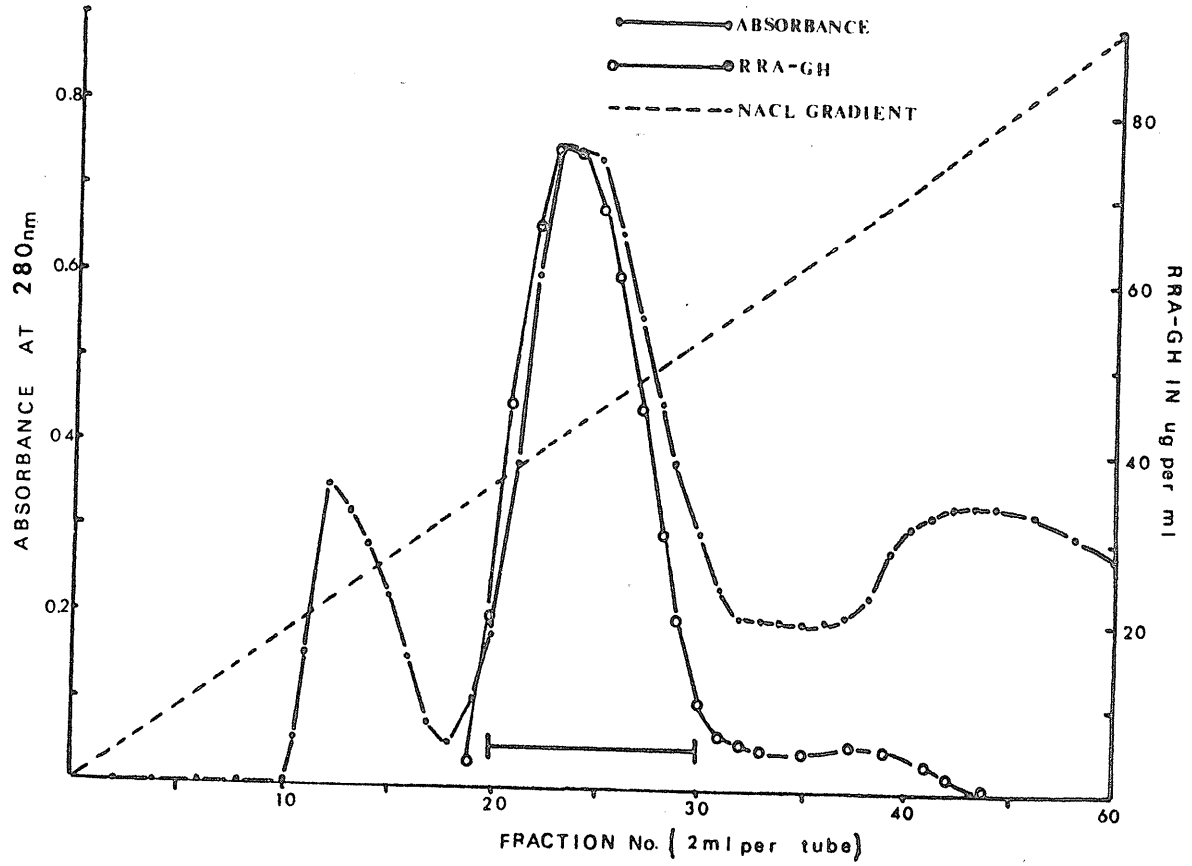

Figure 9. Diethylaminoethyl-Sephadex A-25 column chromatography of the oPL-rich fractions from the Sephadex G-100 column in Fig. 8 (Fractions #42-48). The column (1.2 X 10 cm) was pre-equilibrated with 0.01M Glycine-hydroxide buffer, pH 10.0 . A linear NaCl gradient (0 to 0.05M NaCl) was employed to elute the column.

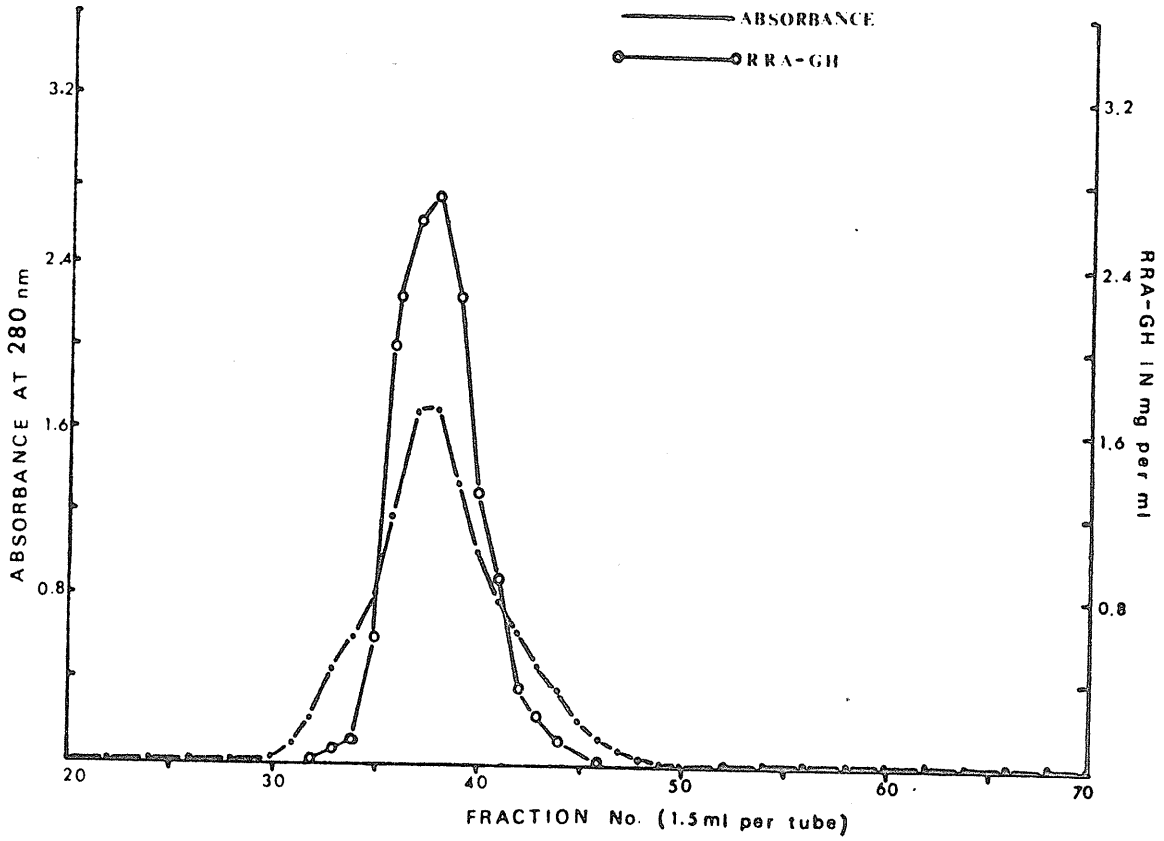

Figure 10. Gel filtration on Sephadex G-50 column (1.4 X 94 cm) of the fractions containing oPL from DE-Sephadex A-25 column (fractions #20-30). The column was equilibrated with 0.1M ammonium bicarbonate, pH 8.2.

TABLE I

TABLE OF PURIFICATION OF OVINE PLACENTAL LACTOGEN

PROCEDURE	PROTEIN* (g)	RRA-GH (mg)	RIA-oPL (mg)	RECOVERY IN % OF PROTEIN	RECOVERY IN % OF RRA-GH	RECOVERY IN % OF RIA-oPL	PURIFICATION FACTOR
Extraction	646	390	312	100	100	100	1
Acidification (pH 6.5)	546	370	294	85	95	94	1
DEAE-Cellulose	150	153	120	23	39	38	2
Acidification (pH 6.0)	103	134	114	16	34	37	3
CM-Cellulose	3.2	67	52	0.5	17	17	34
CM-Sephadex (C-50)	0.65	32.2	27.1	0.10	8.3	8.7	86
Sephadex G-100	0.103	23.8	22.2	0.016	6.1	7.1	446
DE-Sephadex (A-25)	0.021	15.6	17.1	0.0033	4.0	5.5	1685
Sephadex G-50	0.015**	14.7	16.3	0.0023	3.8	5.2	2250

* Protein measured by Lowry method

** Dry weight

N.B. This procedure was employed for the purification of oPL using 3 separate batches of placentaS yielding a total of 35 mg oPL. In general, the results were similar to the data shown above.

Characterization

Analytical Polyacrylamide Gel Electrophoresis

Figure 11 shows the protein pattern of the purified oPL preparation upon electrophoresis run under (a) acidic (pH 4.3-4.5) and (b) alkaline (pH 9.3-9.4) conditions respectively. Under the acidic condition, only one stained band was visible. An unstained gel, in which oPL had been separated in a similar manner, was divided and consecutive gel segments were eluted and assayed for oPL by RRA-GH, GH-like activity was detected in the eluants corresponding to the stained band. However, when it was subjected to polyacrylamide gel electrophoresis at alkaline pH (Fig. 11b), three stained bands were visible. When a duplicate unstained gel was divided and the segments eluted and assayed by RRA-GH, GH-like activities were detected in the eluants corresponding to two stained bands near the anode with the highest GH-like activity associated with the darkest band.

Analytical Gel Isoelectrical-focusing

When the purified oPL preparation was analyzed by analytical gel isoelectric focusing as shown in Figure 12, the oPL preparation displayed two major and one minor bands. When a duplicate gel that was not stained was divided into segments and eluted with buffer, then assayed by RRA-GH as shown in the figure, GH-like activity was found in the eluants coincide with the two bands near the cathode. Similarly, when a highly purified human placental lactogen(hPL,NIH, 95% electrophoretically pure) was subjected to the same run, at least 3 bands were seen.

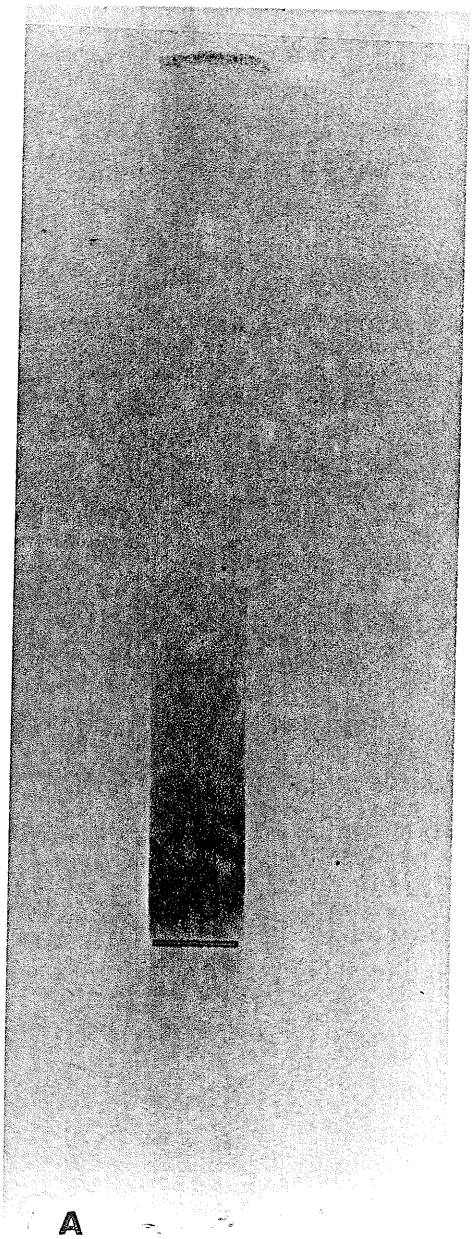

Figure 11. A. Polyacrylamide gel electrophoresis pattern of oPL at pH 4.3-4.5.

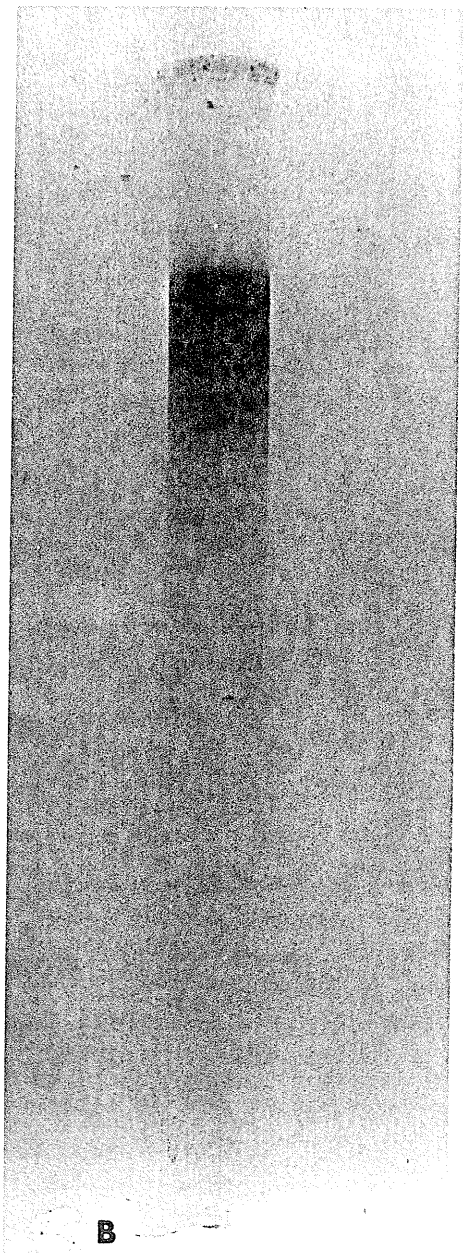

Figure 11. B. Polyacrylamide gel electrophoresis pattern of oPL at pH 9.3-9.5.

Leaf blank to correct

numbering

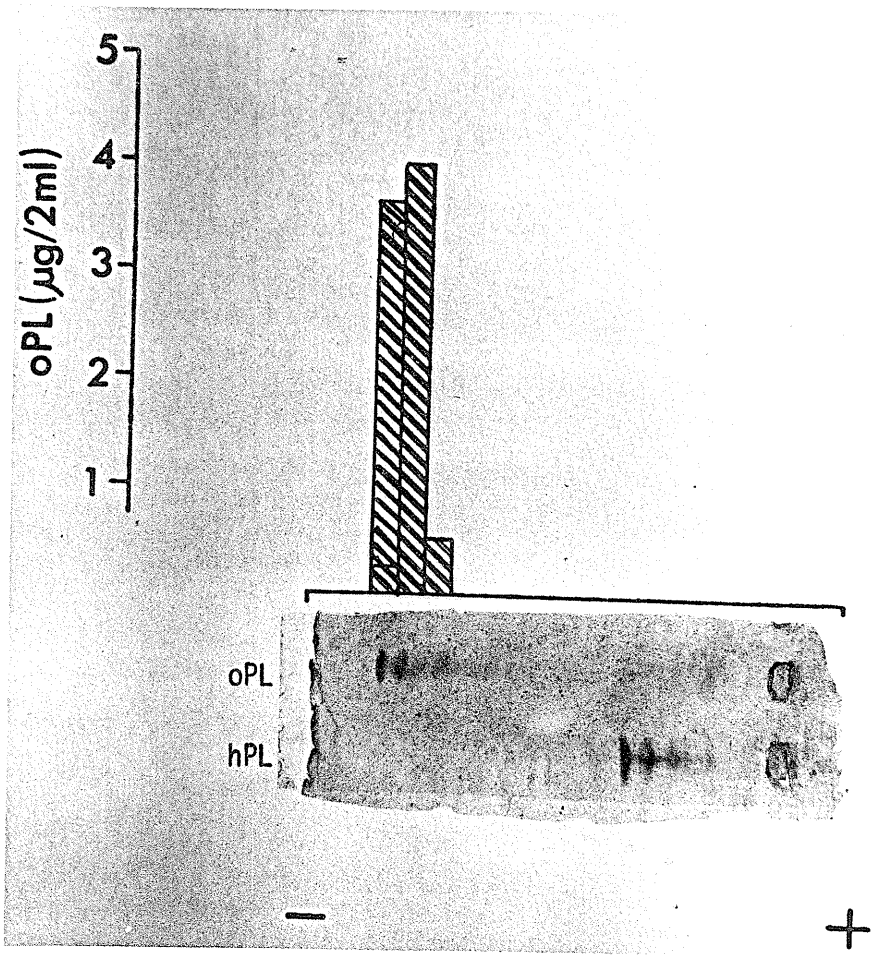

Figure 12. Pattern of oPL activity eluted from polyacrylamide gel isoelectric focusing. Gel eluants were assayed, and the distribution of GH-like activity was determined by RRA-GH. The sample in the upper channel is oPL, whereas the sample in the lower channel is hPL(NIH, 95% electrophoretically pure).

Analytical Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel (Slab) Electrophoresis and Molecular Weight Determination

Figure 13 shows the electrophoretic mobility of the purified oPL (A) in an analytical SDS-polyacrylamide gel (slab) electrophoresis in comparison with other protein markers (B-hPL; C-cytochrome C; D-Ribonuclease; E-Myoglobin; F-Ovine Prolactin; G- Ovalbumin ; H-Chymotrypsinogen A; J-Bovine serum albumin).

When the distance traveled by these proteins is plotted against the known molecular weight of the proteins as shown in Figure 14, it is apparent that the molecular weights of oPL, hPL, and oPRL are very similar, estimated between 19,500 to 21,500.

Displacement Curves of oPL in the Radioreceptorassay for Prolactin (RRA-PRL) and for Growth Hormone (RRA-GH) using Rabbit Tissues

When the purified oPL preparation was assayed in both the RRA-PRL and RRA-GH (Figure 15 a and b respectively), oPL inhibited the binding of either ^{125}I-oPRL or ^{125}I-oGH in a parallel manner to the hormone standards used (oPRL for RRA-PRL and oGH for RRA-GH respectively). In the RRA-PRL, oPL has twice the potency of oPRL. Whereas in the RRA-GH, oPL is about equal potent with oGH in competing for GH-receptor sites. When the ratio of prolactin to growth hormone

activity of oPL was compared with that found for hGH and hPL by the two radioreceptorassays, it is apparent that oPL and hGH have a ratio of 2:1, whereas hPL has a ratio of 100:1.

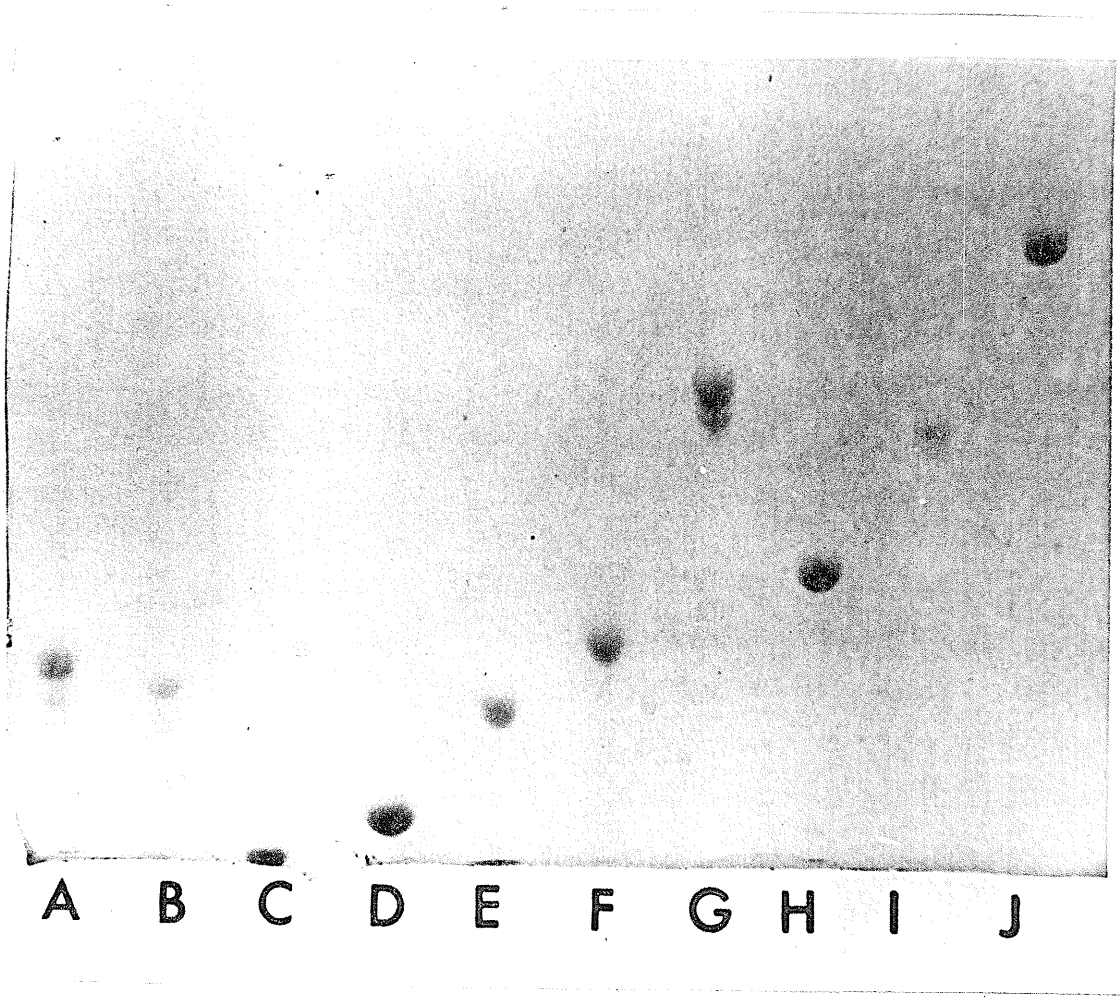

Figure 13. The electrophoretic mobility upon sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of A-oPL, B-hPL, C-Cytochrome C, D-Ribonuclease, E-Myoglobin , F-Ovine prolactin, G-Ovalbumin, H-Chymotrypsinogen A, and J-Bovine serum albumin.

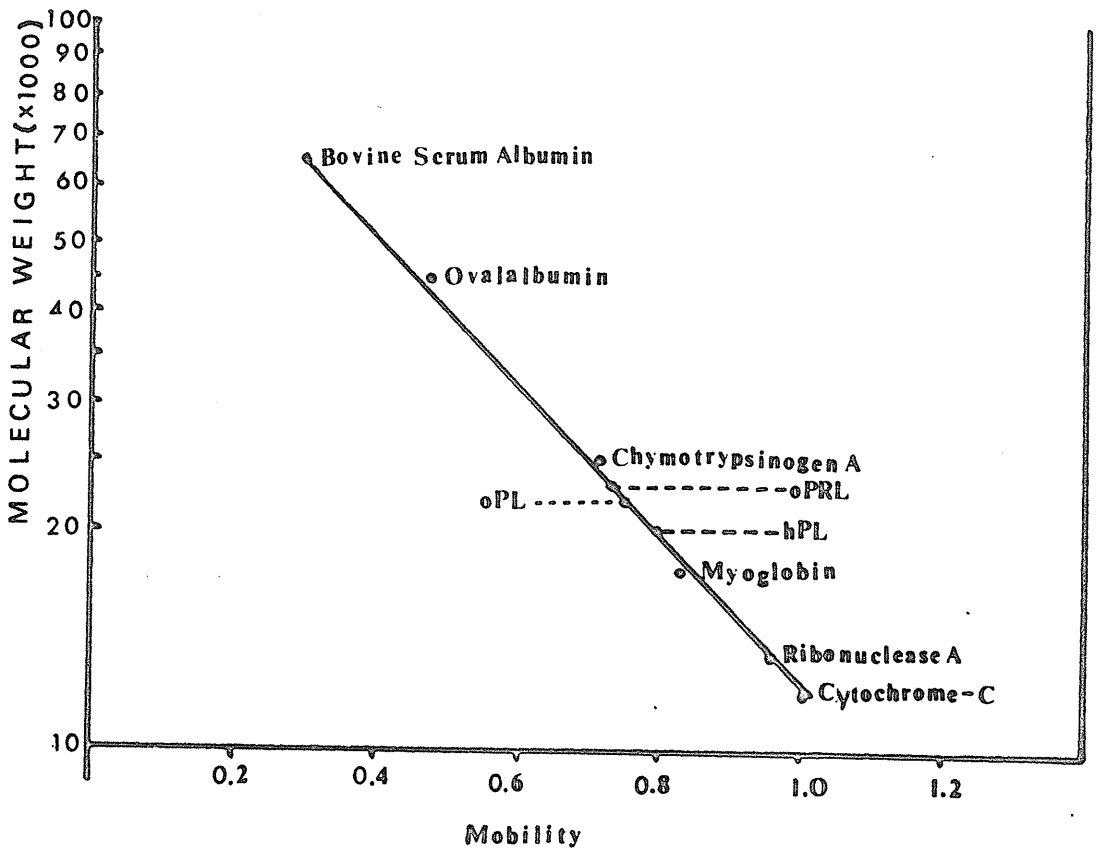

Figure 14. The relative mobility of different proteins after acrylamide gel electrophoresis in gels containing sodium dodecyl sulfate, plotted against the logarithm of the molecular weight of proteins. oPL has a molecular weight between that of hPL and oPRL.

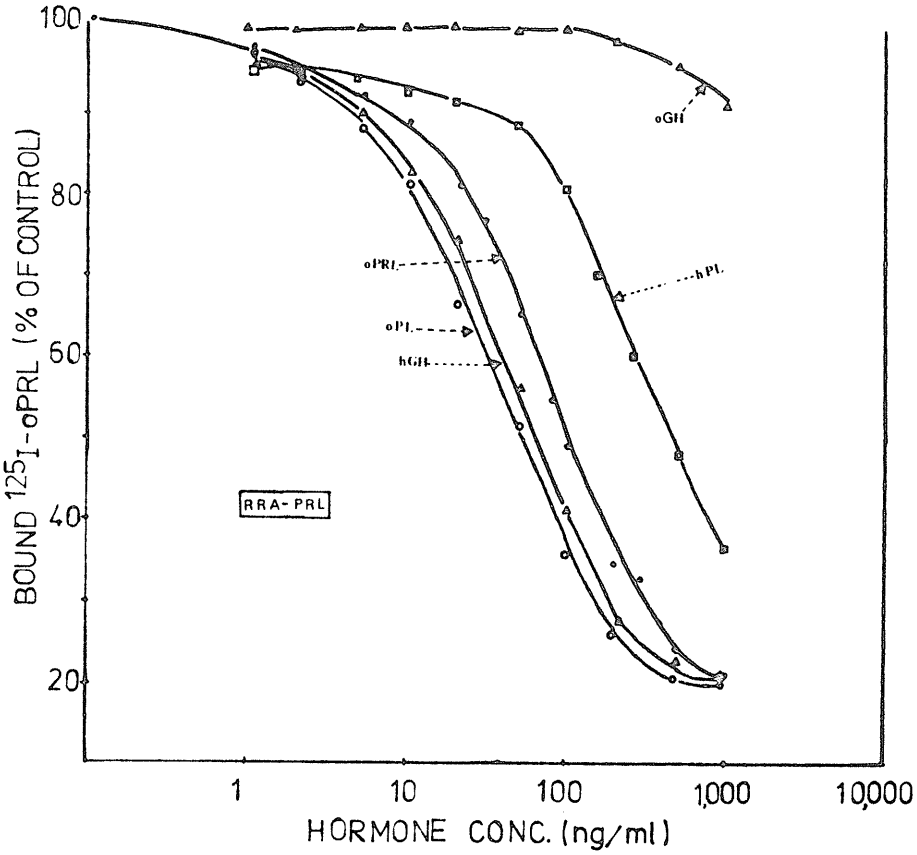

Figure 15a. Displacement curve for oPL, hGH, oPRL, hPL, and oGH in the radioreceptorassay for prolactin(RRA-PRL) using rabbit mammary gland. Rabbit mammary gland receptors were incubated with ^{125}I-oPRL in the presence of increasing concentrations of"cold" hormone. The ordinate represents the ^{125}I-oPRL bound to prolactin binding sites. In the absence of any added hormone, the amount bound is taken to be 100%. In the presence of oPRL, oPL, hGH, and hPL, the % of ^{125}I-oPRL bound is decreased. The abscissa represents the concentration of hormone added to the assay tube.

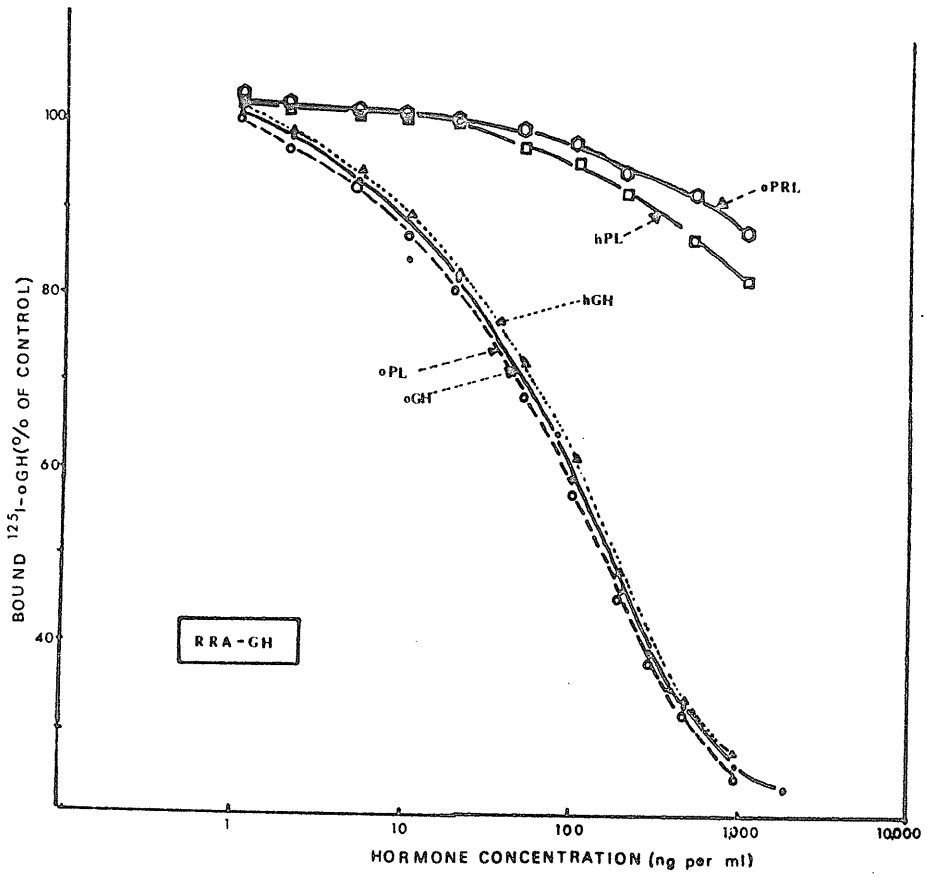

Figure 15b. Displacement curve for oPL, hGH, hPL, oPRL, and oGH in the radioreceptorassay for growth hormone (RRA-GH) using rabbit liver. Rabbit liver (100,000 Xg fractions) was incubated with ^{125}I-oGH in the presence of increasing concentrations of oGH, oPL, oPRL, and hPL. The ordinate represents the displacement of ^{125}I-oGH bound to growth hormone binding sites in the absence and presence of oGH, hGH, oPRL, and hPL. The amount of ^{125}I-oGH bound in the absence of "cold" hormone is taken to be 100%. The abscissa represents the concentration of "cold" hormone added.

Radioreceptorassay for Ovine Placental Lactogen using Human Liver

When human liver membrane preparations were used for the radioreceptorassay as shown in Figure 16, the purified oPL preparation was twice as potent as hGH in inhibiting the binding of ^{125}I-oPL, whereas non-primate GH preparations(oGH and bGH not shown) failed to inhibit even at concentration(10 ug/ml). Similarly,ovine prolactin also was without effect in the assay. In this assay hPL caused a slight inhibition only at concentrations in excess of 1,000 ng/ml and hence, it's cross-reaction in this assay is less than 1%. These results (data from Figure 15 and 16) suggest a close structural homology between oPL and primate GH.

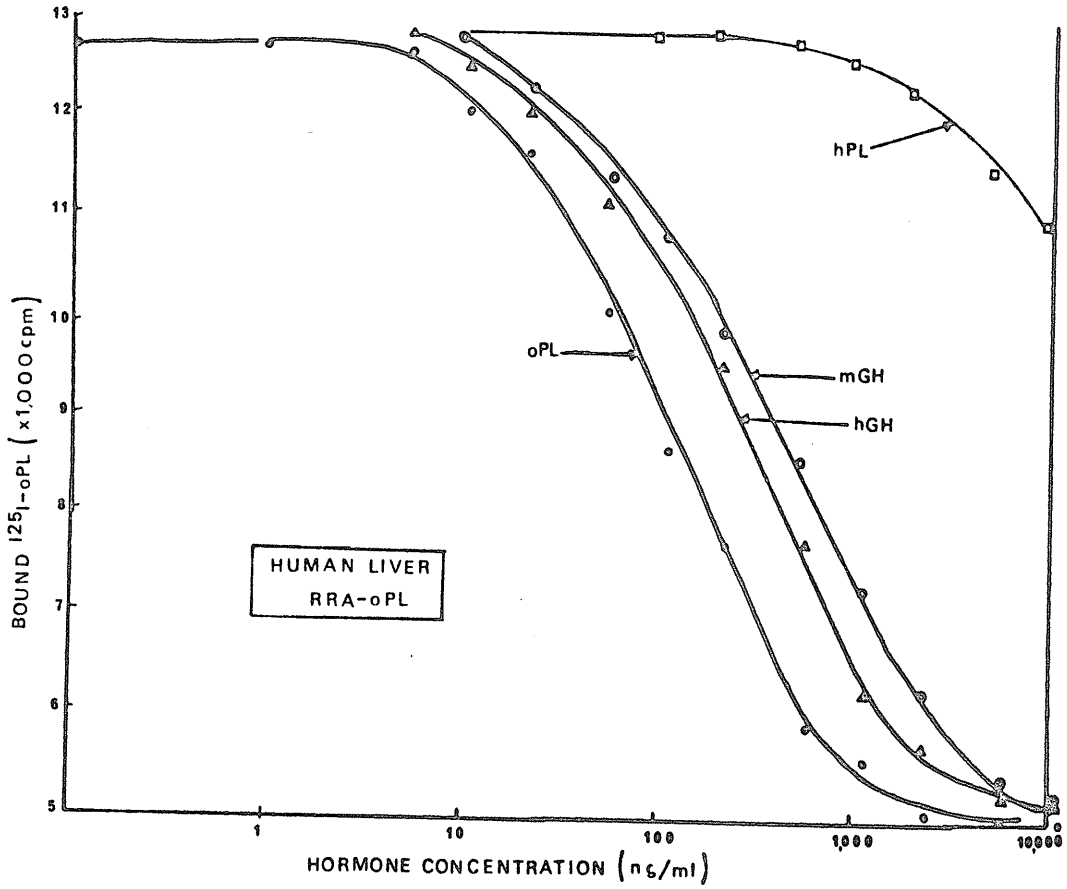

Figure 16. Radioreceptorassay for ovine placental lactogen using human liver (100,000 x g fractions). The ordinate indicates the cpm of ^{125}I-oPL bound to the human growth hormone binding sites in the absence or presence of "cold" hormone added to the assay tube.

B: DETECTION AND CHARACTERIZATION OF THE RECEPTOR FOR OVINE PLACENTAL LACTOGEN IN THE SHEEP

Tissue Survey

The results of the tissue survey are shown in Table II. High specific binding of oPL to 100,000 x g pellet was found for the liver, adipose tissue, ovary, corpus luteum, and uterus of the ewe, and the fetal liver. In the remaining tissues, specific binding was less than 3%.

Effect of Protein Concentration on the Specific Binding of ^{125}I-oPL to different Tissues

Figure 17 shows that in 100,000 x g fractions from several tissues specific binding of oPL increased as the amount of protein increased.

Effect of Incubation Time and Temperature on the Binding of Ovine Placental Lactogen to Different Tissues

Figure 18A shows the effect of incubation time and temperature on the specific binding of oPL to 100,000 x g pellets obtained from liver and adipose tissue of the non-pregnant ewe and from corpora lutea of pregnant ewes. Specific binding was maximal at 24 h for all tissue binding sites when the incubation was performed at 4 C. Figure 18B depicts similar results when 100,000 x g pellets from the ovary and uterus of the non-pregnant ewe and the liver of a fetus obtained

TABLE II

Percent specific binding of ovine [^{125}I]iodo-PL in tissue preparations from ewe and fetus[a]

Tissue used for binding studies	Ewe			Fetus (130–145 days)
	Non-pregnant	Pregnant (days)		
		22–27	130–135	
Liver	28 ± 5.0 (5)[b]	30 ± 4.0 (7)	25 ± 7 (3)	5.0 ± 2 (3)
Adipose tissue	20 ± 4.4 (7)	18 ± 5 (7)	10 ± 5 (3)	1.4 ± 0.9 (3)
Ovary	9.1 ± 2[c] (3); 10.2 ± 1[d] (2)	7 ± 1.5 (6)	6.5 ± 1.3 (7)	
Corpus luteum	6.1 ± 5[d] (3)	6.2 ± 2.8 (12)	5.0 ± 2 (3)	
Uterus	5 ± 2 (5)	4.2 ± 1.7 (4)	1.5 ± 1 (3)	
Mammary gland	2.5 ± 1.3 (3)	1 ± 0.4 (5)	0.5 ± 0.5 (3)	
Adrenal	0.5 ± 0.2 (2)		0.7 ± 0.2 (3)	0.5 ± 0.4 (3)
Kidney	1.9 ± 1 (3)	0.9 ± 0.4 (2)	1.0 ± 0.4 (5)	1.7 ± 1 (3)
Spleen	0 ± 0.2 (3)	1.8 ± 0.5 (3)	0.9 ± 0.4 (2)	0.6 ± 0.4 (3)
Pancreas	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (3)	0.7 ± 0.5 (2)
Lung	1.1 ± 0.1 (2)	1.5 ± 0.5 (3)	0.9 ± 0.5 (3)	1.3 ± 0.5 (3)
Heart	0.6 ± 0.3 (2)	0.4 ± 0.2 (4)	0.5 ± 0.4 (3)	1.2 ± 0.3 (3)
Skeletal muscle		0.3 ± 0.5 (5)	0.4 ± 0.3 (3)	1.3 ± 0.4 (3)
Brain (cortex)			0.5 ± 0.1 (2)	1.5 ± 0.2 (3)
Maternal caruncles	3.7 ± 0.5 (2)		0.5 ± 0.2 (2)	
Fetal cotyledons			0.2 ± 0.2 (2)	

[a] An incubation time of 24 h at 4 C was used for all tissues. The percentage (± SD) of specific binding of ovine [^{125}I]iodo-PL is expressed as per 1,000 ± 50 μg protein.

[b] The numbers in parentheses represent the number of ewes studied.

[c] The ewes were killed during anestrus.

[d] The ewes were killed during the estrous cycle.

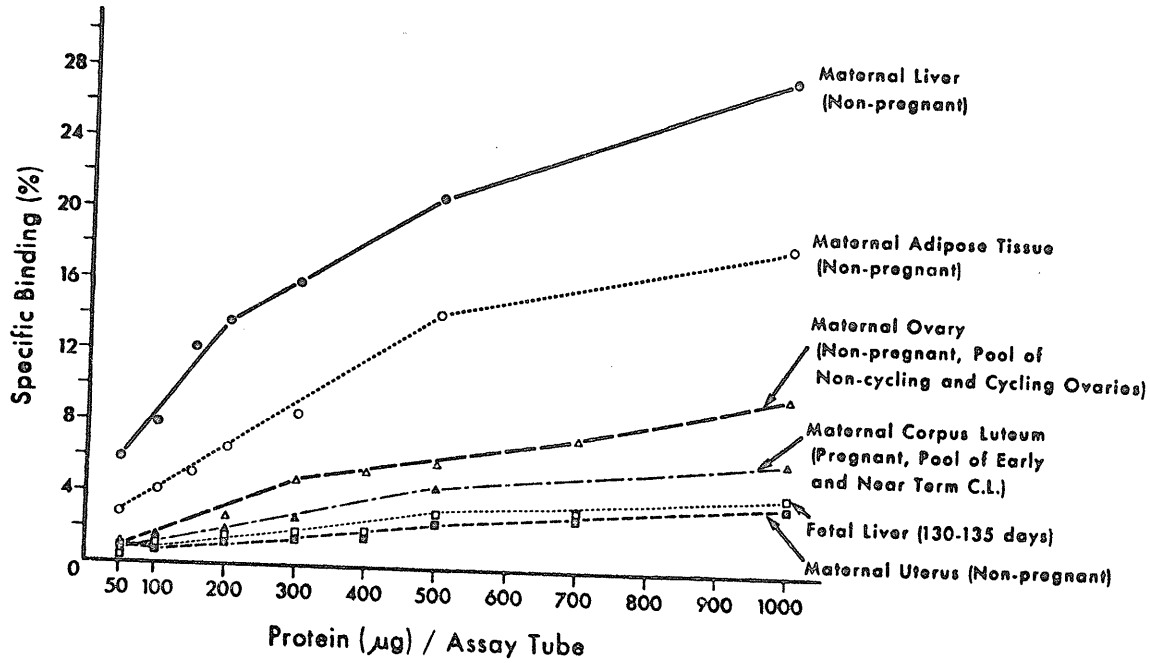

Figure 17. Effect of protein concentration (100,000 x g fraction) on the specific binding of ^{125}I-oPL to different tissues.

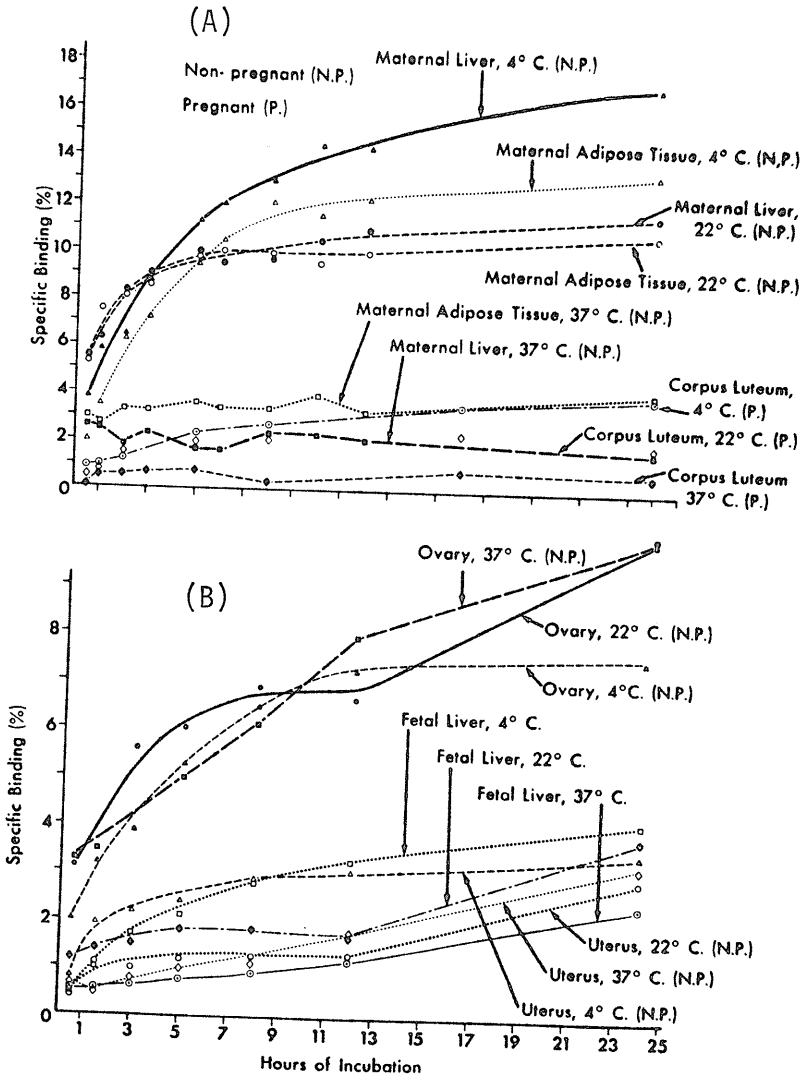

Figure 18. A. Effect of incubation time and temperature on the binding of ^{125}I-oPL to 100,000 x g pellets of non-pregnant ewe liver, and adipose tissue as well as corpora lutea removed from ovaries which were obtained both early and late in pregnancy. B. Effect of incubation time and temperature on thebinding of ^{125}I-oPL to 100,000 x g pellets from non-pregnant ewe ovary (pool of estrous and anestrous ovary) and uterus, and fetal liver (130-135 days of gestation).

late in pregnancy were used. Again maximal binding of oPL was obtained after incubation at 4 C for 24 h, with the exception of the ovary, where binding of oPL at 22 C or 37 C was slightly greater. During routine assays, 24 h incubation periods at 4 C were used for all tissue receptors because this gave the highest level of binding within a convenient time period.

Radioreceptorassay for oPL using Sheep Liver and Adipose Tissues

Figure 19 shows the competition by pituitary hormones for oPL binding sites in liver and adipose tissue obtained from the non-pregnant ewe. Pituitary growth hormones from various species competed with oPL for binding sites in liver to varying degrees(Fig. 19A). Human placental lactogen competed only minimally and other pituitary hormones not at all. Similarly, the results in Fig. 19B for adipose tissue(100,000 x g fractions) show that growth hormones inhibited the binding of the tracer in a parallel manner to that of oPL except that 2 to 5 fold increases in concentration were required. Scatchard analysis of the data obtained with oPL are displayed in the figure and the number of binding sites (n) and the affinity constant (Ka) of oPL for each tissue is indicated.

Radioreceptorassay for oPL using Sheep Corpus Luteum and Ovary

Figure 20 shows the inhibition of binding of ^{125}I-oPL to the corpus luteum and to the ovary (100,000 x g fractions) by oPL and three ovine pituitary hormones (PRL, GH, and LH). Corpora lutea

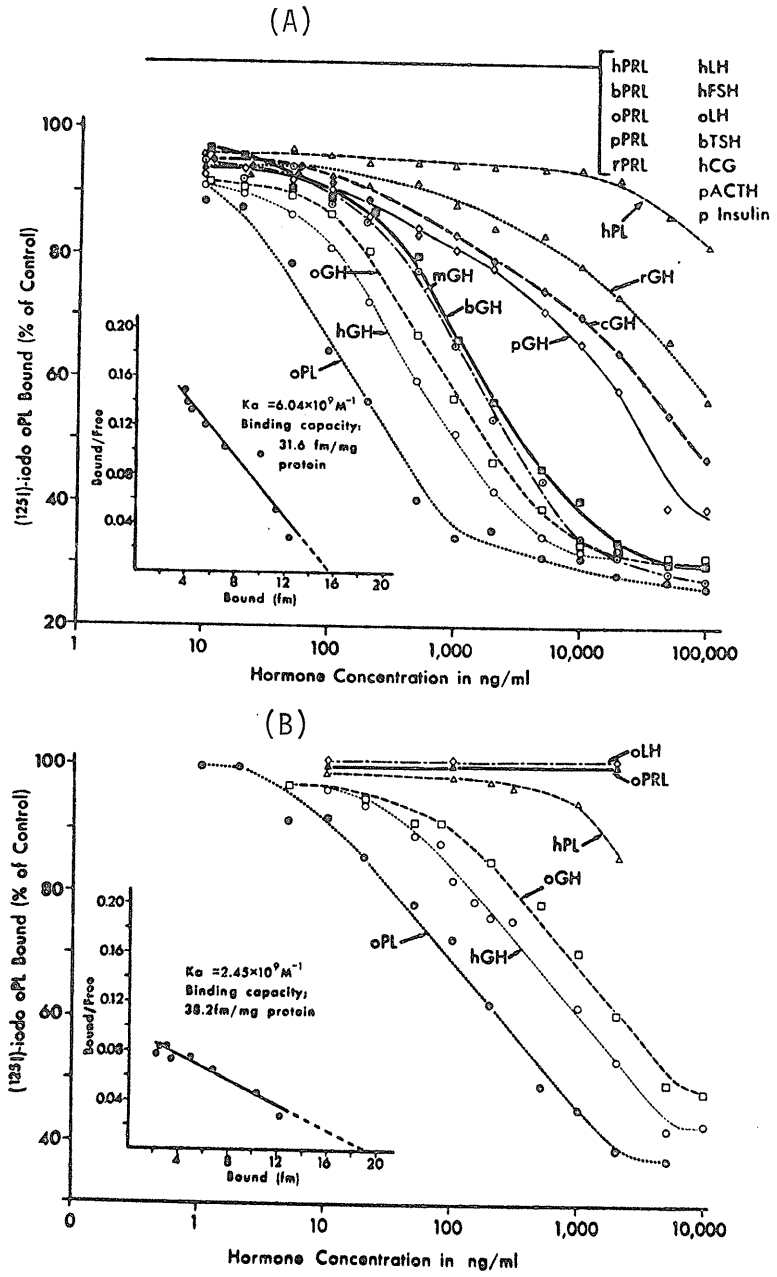

Figure 19. Radioreceptor assay for oPL using pellets (100,000 x g fractions) from the liver(A) and adipose tissue(B) of non-pregnant ewes, using 500 ug protein per assay tube. The amount of radioactivity bound in the absence of oPL was taken to be equal to 100%. The specific binding of ^{125}I-oPL to liver and adipose tissue was 22% and 16%, respectively. The inset depicts a Scatchard analysis of the data.

were obtained from pregnant and non-pregnant ewes. Fig. 20A shows that oGH inhibited the binding of oPL to corpus luteum in a parallel manner, whereas, oPRL and oLH did not inhibit. Figure 20B shows that in the ovary, oPRL and oLH slightly inhibited the binding of oPL in a non-parallel manner, and oGH caused an inhibition of 20-30% which is at least 10% lower than that observed in other tissues. Scatchard plots of data obtained again are shown.

Radioreceptorassay for oPL using Non-pregnant Sheep Uterus and Fetal Liver

Figure 21 shows the inhibition of binding by ovine PL, GH, PRL, and LH to uterine preparations (obtained from non-pregnant ewes) and to the preparations of fetal liver obtained from a mature fetus at 130-135 days of gestation. In both tissues (figure 21A for uterus and B for fetal liver), only GH inhibited the binding of PL whereas PRL and LH did not.

Radioreceptorassay for oPRL using Pregnant Sheep Uterus

We have studied the binding of ovine prolactin to 780 x g, 15,000 x g, and 100,000 x g fractions of various ovine tissues. Specific binding of ^{125}I-oPRL was less than 3% / mg protein in all fractions of the following tissues from the ewe (both pregnant and non-pregnant): mammary gland, adrenal, kidney, spleen, pancreas, lung, heart, skeletal muscle, brain(cortex), and uterus(non-pregnant and 20-60 days of gestation). Less than 3 % specific binding was also found in several fetal tissues

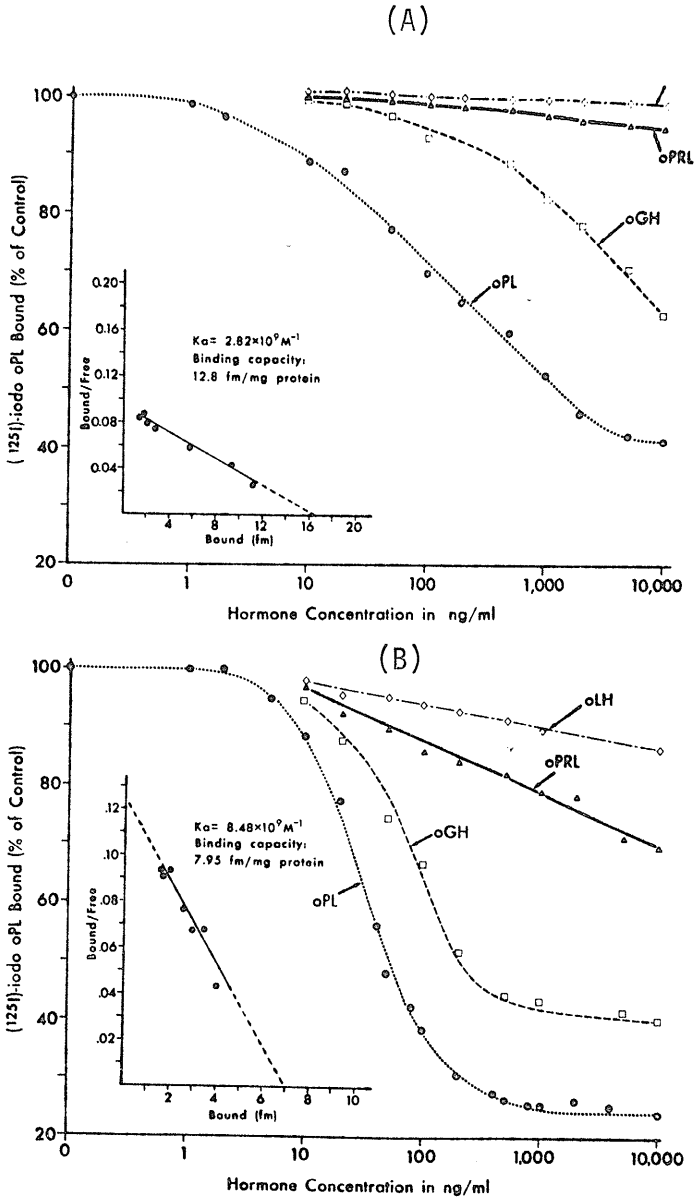

Figure 20. Radioreceptor assay for oPL using pellets (100,000 x g fractions) obtained from the corpora lutea of pregnant ewes of early and late gestational period and the ovaries of non-pregnant ewes. A. Corpus luteum, 1.5 mg protein was used per assay tube, and B. Ovary, 1.0 mg protein was used. The percentage of specific binding of ^{125}I-oPL was 11% and 17.3%, respectively.

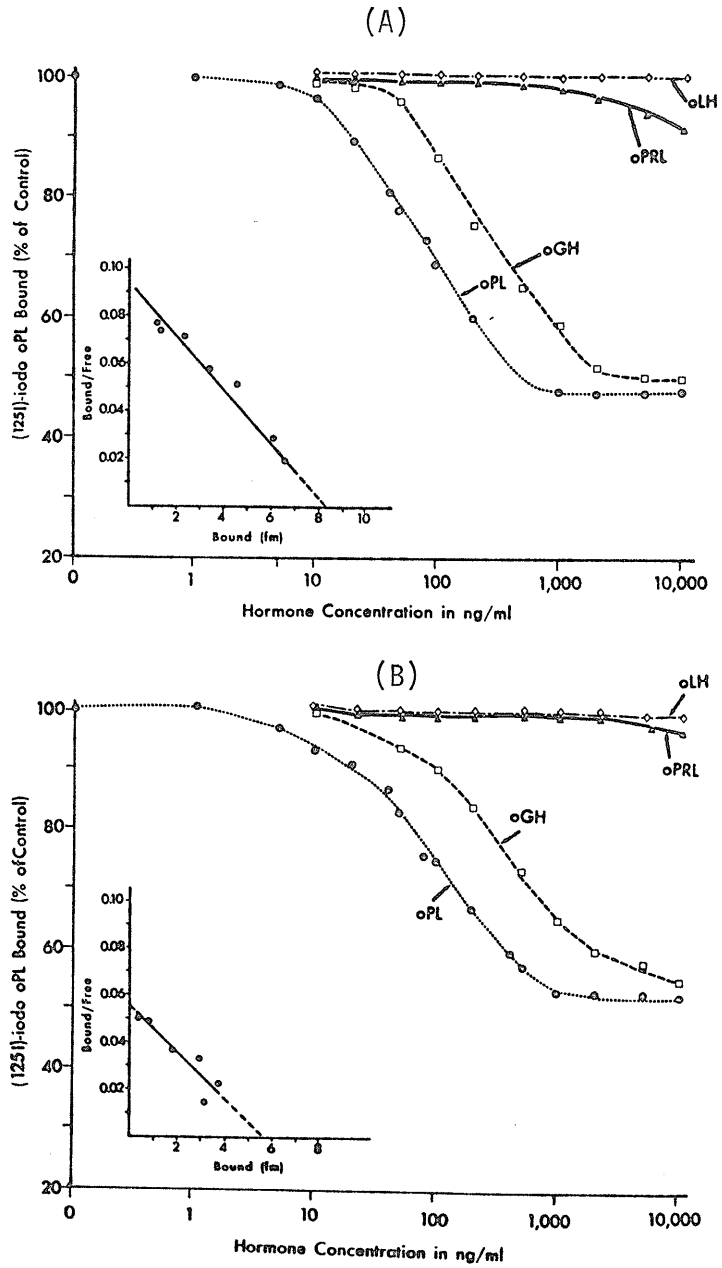

Figure 21. Radioreceptor assay for oPL using pellets (100,000 x g fractions) from non-pregnant uterus (A) and fetal liver (B). In both panels, 1.5 mg protein was used per assay tube, and the percentage of specific binding of ^{125}I-oPL was 13.2% and 10.4%, respectively. The Ka and N for uterus are 5.35 X $10^{9}M^{-1}$ and 5.5 fmol/mg protein, respectively, and for fetal liver, 5.05 X $10^{9}M^{-1}$ and 3.7 fmol/mg protein, respectively.

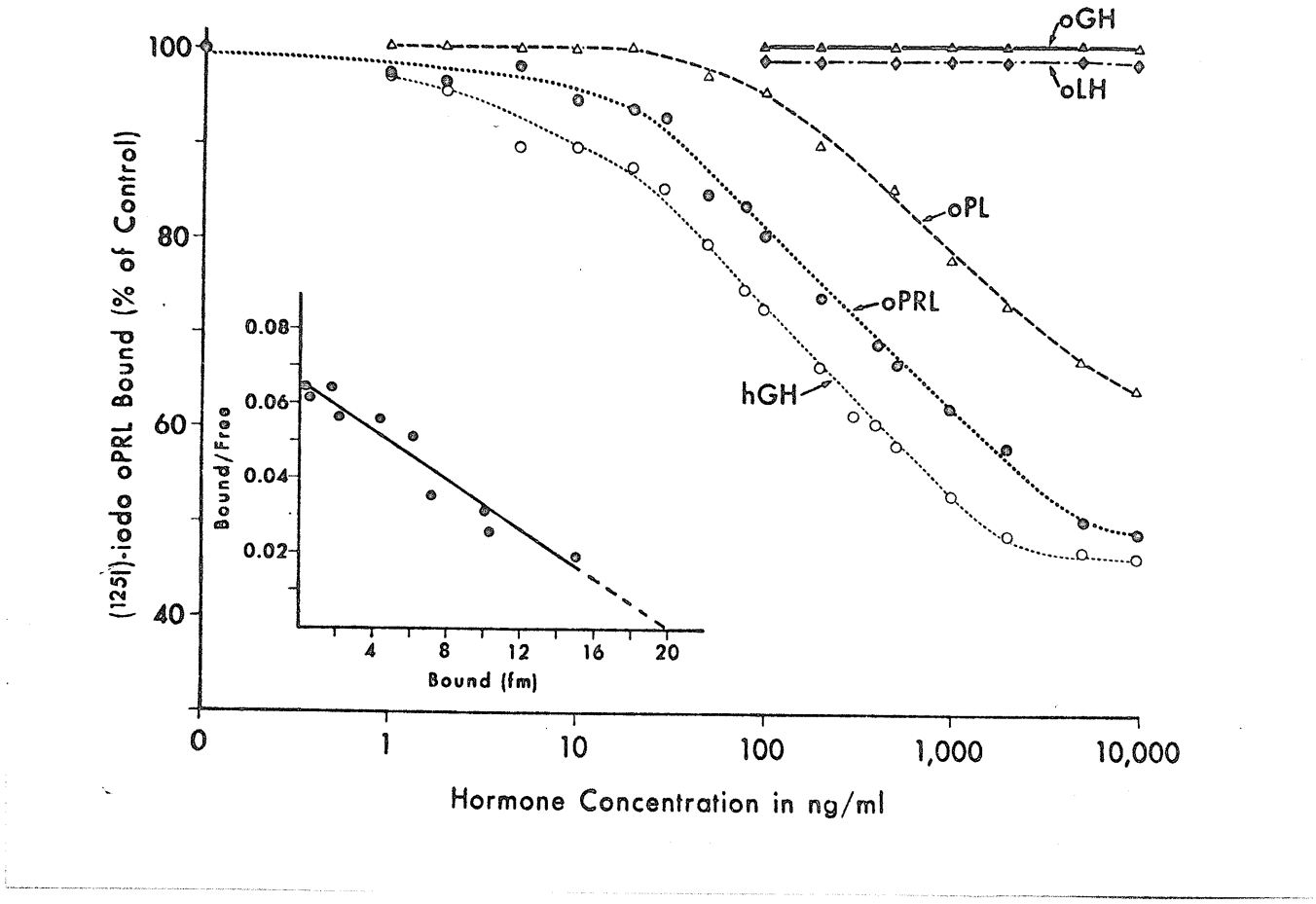

Figure 22. Radioreceptor assay for oPRL using pellets (100,000 xg fractions) obtained from the uterus of the pregnant ewe (90-135 days of gestation). 500 ug protein was used per assay tube, and the percentage of specific binding of ^{125}I-oPRL was 10.8%. The Ka and N for uterus are 1.65 X $10^{9}M^{-1}$ and 40 fmol/mg protein, respectively.

including adipose tissue, adrenal, kidney, spleen, pancreas, lung, heart, skeletal muscle, brain(cortex) and placental tissue near term (both fetal and maternal cotyledons). The specific binding of ^{125}I-oPRL to 100,000 x g pellet obtained from pregnant sheep uterus (90-135 days) increased to 14 ± 3.6% / mg protein (average of studies in uterine tissue from six pregnant ewes) and less than 3% in the 780 x g and 15,000 xg pellets from these animals. As shown in Figure 22, oPL was only 1/10 as active as oPRL in competing for the PRL binding sites, whereas hGH was at least twice as potent as the PRL standard.

Comparative Studies on the Placental Lactogen-like, Growth Hormone-like, and Prolactin-like activities in the Placental Extracts of Various Species

Figure 23 depicts the hormonal activities (placental lactogen-like, A; growth hormone-like, B; and prolactin-like, C) in the placental extracts of various species. When the concentrations of these hormonal activities for various species are calculated using oPL, hGH, and oPRL as standards as shown in Table III, it appears that sheep placental extract has the highest placental lactogen-like activity, monkey placental extract has the highest growth hormone-like activity, and primate(human and monkey) placental extracts contain the highest prolactin-like activity.

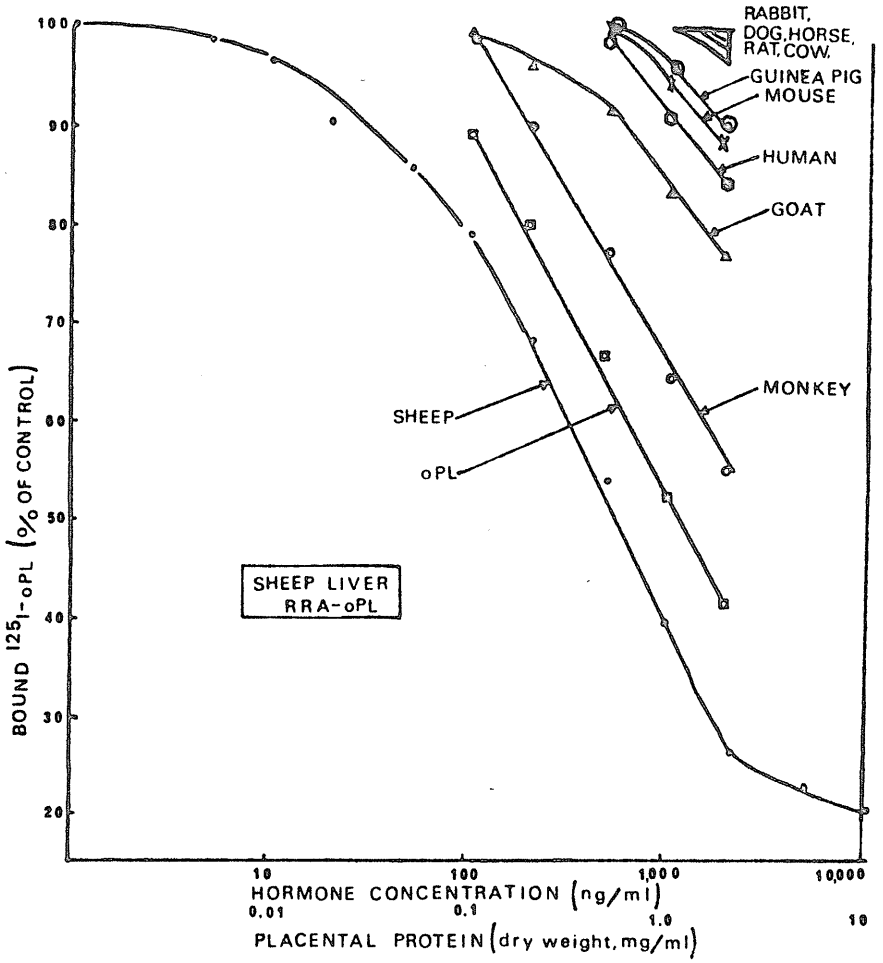

Figure 23A. Estimation of placental lactogen-like activity in the placental extracts of various species using oPL and sheep liver (100,000 x g fractions) as hormone standard and receptors respectively.

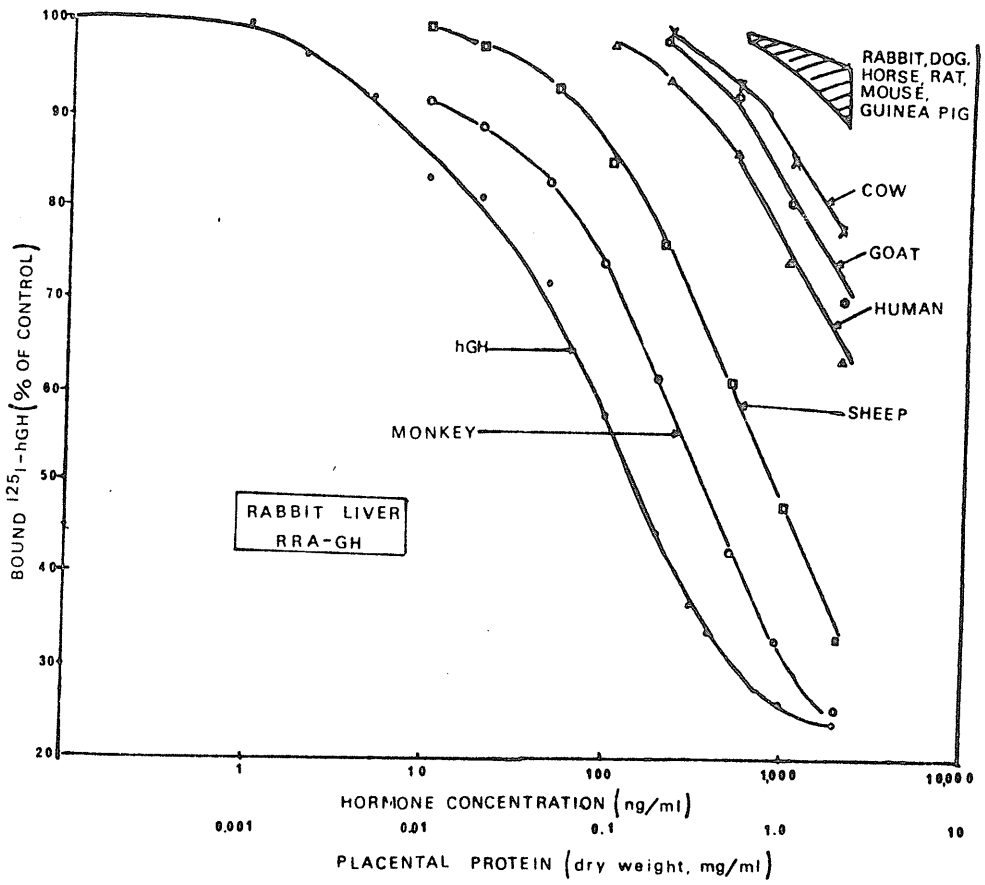

Figure 23B. Estimation of growth hormone-like activity in the placental extracts of various species using hGH and rabbit liver (100,000 x g fractions) as hormone standard and receptors respectively.

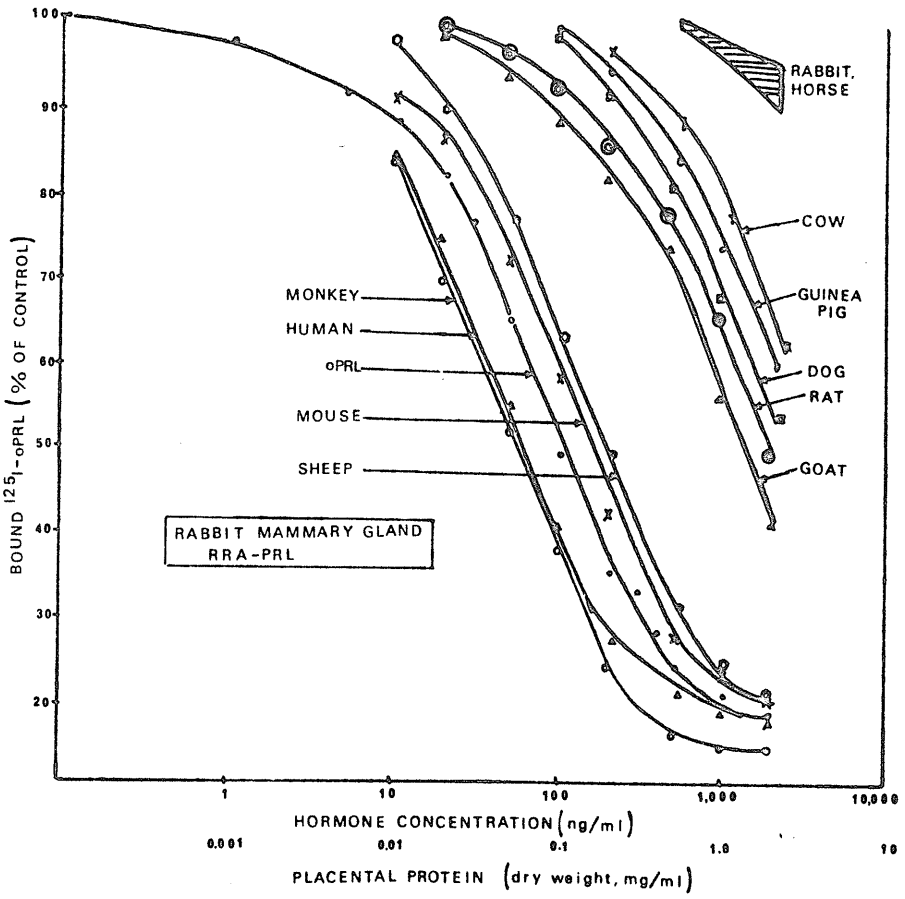

Figure 23C. Estimation of prolactin-like activity in the placental extracts of various species using oPRL and rabbit mammary gland (100,000 X g fractions) as hormone standard and receptors respectively.

TABLE III

PLACENTAL LACTOGEN-LIKE*, GROWTH HORMONE-LIKE**, AND PROLACTIN-LIKE*** ACTIVITIES IN THE PLACENTAL EXTRACT OF VARIOUS SPECIES

SPECIES	DAYS OF GESTATION	PL-LIKE (ng/mg)	GH-LIKE (ng/mg)	PRL-LIKE (ng/mg)
Sheep	130 (term at 144-152 days)	500	200	550
Monkey	160 (term at 168 days)	200	500	1600
Goat	140 (term at 145-151 days)	50	22	80
Human	270 (term at 270-290 days)	25	35	1600
Mouse	19 (term at 19-20 days)	25	<5	700
Guinea pig	60 (term at 68 days	17	<5	30
Dog	54 (term at 61 days)	<5	<5	45
Cow	255 (term at 277-290 days)	<5	10	25
Rat	19 (term at 22 days)	<5	<5	50
Horse	331 (term 330-345 days)	<5	<5	<5
Rabbit	29 (term 31 days)	<5	<5	<5
Rabbit(kidney)	29 (term 31 days)	<5	<5	<5
Rabbit(lung)	29 (term 31 days)	<5	<5	<5

* Purified oPL as standard. **hGH(NIH-HS 1648E, 2U/mg) as standard.

** * oPRL(NIH-P-S-10, 26 IU/mg) as standard.

C: DEVELOPMENT OF A SPECIFIC AND SENSITIVE RADIOIMMUNOASSAY FOR oPL

Standard Curve of oPL in the Radioimmunoassay(RIA)

Figure 24 shows the sensitivity and specificity of the RIA for oPL. Ovine pituitary GH(oGH) and ovine PRL(oPRL) do not cross-react (not shown). Similarly, several hormone preparations from several species either of pituitary or placental origin showed no cross-reaction(not shown). The only samples which cross-reacted in the assay were pregnant sheep placental extracts (after 20 days of gestation), fetal serum (not shown), and pregnant sheep serum. These results indicate that oPL is immunologically different from sheep pituitary GH and PRL and also a variety of other hormone preparations. The intraassay(three pregnant sheep plasma samples assayed 10 times in the same assay) and interassay (six pregnant sheep plasma assayed in five consecutive assays) coefficients of variation were 10.5% and 8.2%, respectively.

The hormone preparations tested were hGH(NIH 2019G,2.2 IU/mg), mGH(NIH M-945A,0.9IU/mg), bGH(NIH B-1003A, 2.0IU/mg),oGH(NIH O-743B, 1.2 IU/mg), bPRL(NIH P-B_3, 18IU/mg), oPRL(NIH-P-S-10,26IU/mg), hPL(NIH, 95% electrophoretically pure), hPRL, mPL, rPL, cPL, and bPL(all placental lactogens were purified in our laboratory; each have 10-30% potency of the oPRL standard when assayed in the RRA-PRL).These hormones were tested at concentrations as high as 10 ug/ml.

The placental extracts tested were human, monkey, cow, horse, dog, rabbit, rat, mice, hamster, and guinea pig. Similarly, pituitary extracts of these species were also tested.

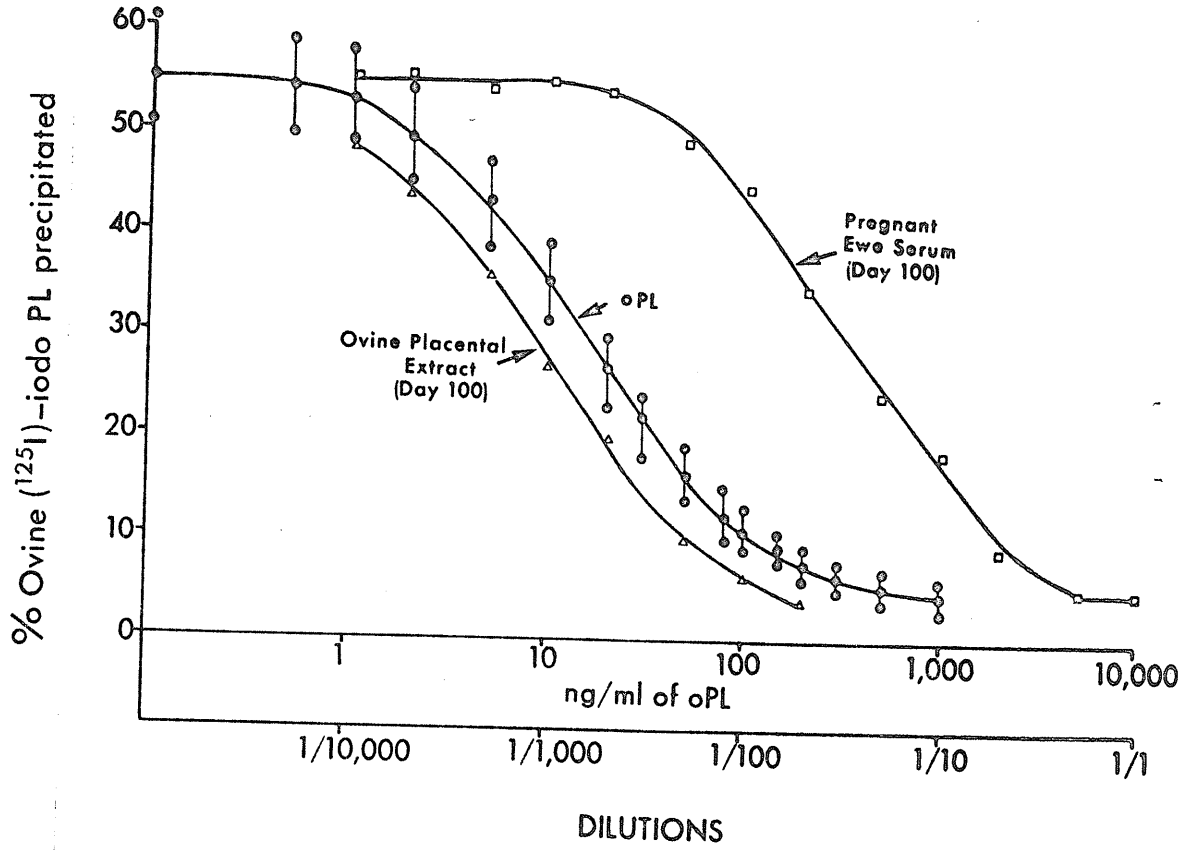

Figure 24. Comparison of dilution curves for pregnant sheep serum (□-----□) and placental extract (Δ-----Δ) (100 days of gestation) with oPL standard (o-----o) in the RIA for oPL. The oPL standard curve represents the mean ± SD of six assays using several different ^{125}I-oPL preparations. There was no cross-reaction with extracts of ovine caruncle and sera obtained from non-pregnant ewes (not shown).

D. STUDIES ON THE SECRETION OF oPL DURING PREGNANCY

(1) Studies on the Maternal and Fetal Secretion of oPL during Pregnancy

Maternal and Fetal Concentrations of oPL

Figure 25 shows the concentration of plasma oPL in 11 pregnant ewes at various stages of pregnancy up to 141 days of gestation. Parturition occurred at day 141-150. After day 48, oPL concentrations were greater than 2 ng/ml and increased as pregnancy advanced to peak levels on day 131-141. The increased variability at day 141 no doubt reflects the differences in the time at which the preparturition drop commences.

After the initial peak of oPL at day 131-141, there was generally a decline in PL concentration commencing approximately 5 days before parturition as shown in Figure 26. In the postpartum period, oPL levels decreased rapidly.

Comparison of Uterine and Jugular Vein Serum oPL Concentration during Pregnancy

Figure 27 depicts the pattern of oPL detected in uterine vein blood and in peripheral blood. By day 25, oPL was detectable in uterine vein blood of some animals. In this study, it was found that oPL levels in uterine blood were consistently higher than those in the peripheral blood in the same ewe.

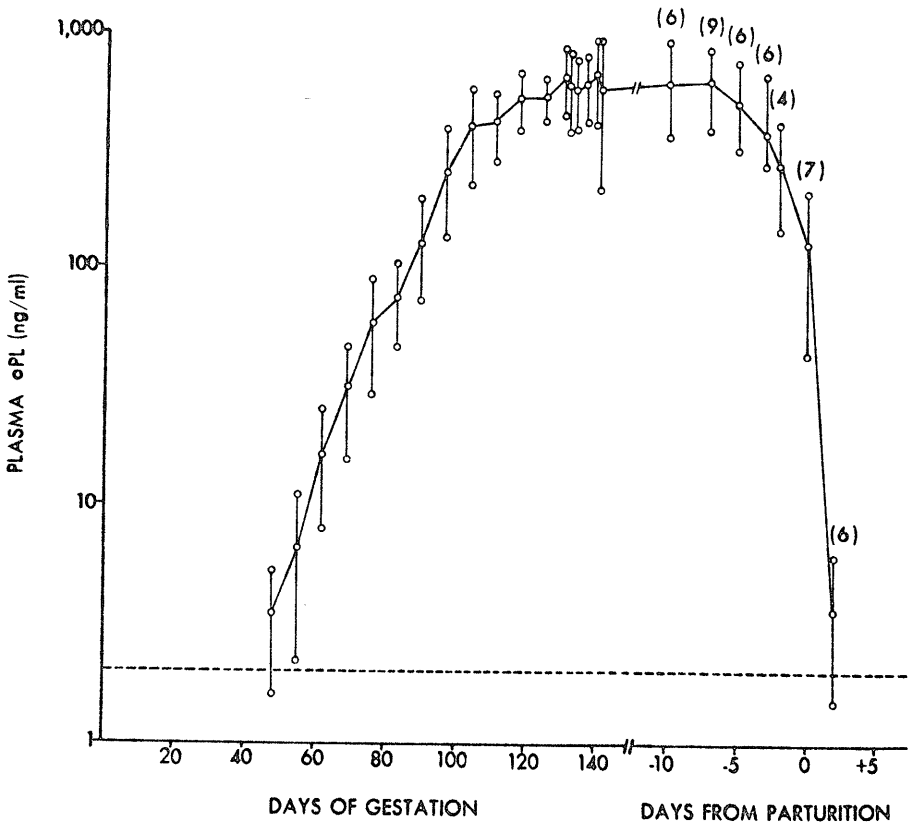

Figure 25. Plasma oPL concentrations during pregnancy measured by RIA. Six of eleven sheep studied were singleton gestations and the remaining five sheep were twin gestations. Vertical lines indicate ± SD of 11 sheep except where indicated. All animals studied had a normal pregnancy. One delivered at day 141, three ewes delivered at day 144, two ewes delivered at day 145, two delivered at day 146, and the remaining three ewes delivered a t 150 days of gestation. The dotted line in this and subsequent figures indicates the sensitivity of the assay. The decline of plasma oPL concentrations before and after parturition also indicated on the right. The number in brackets represents the number of animals studied.

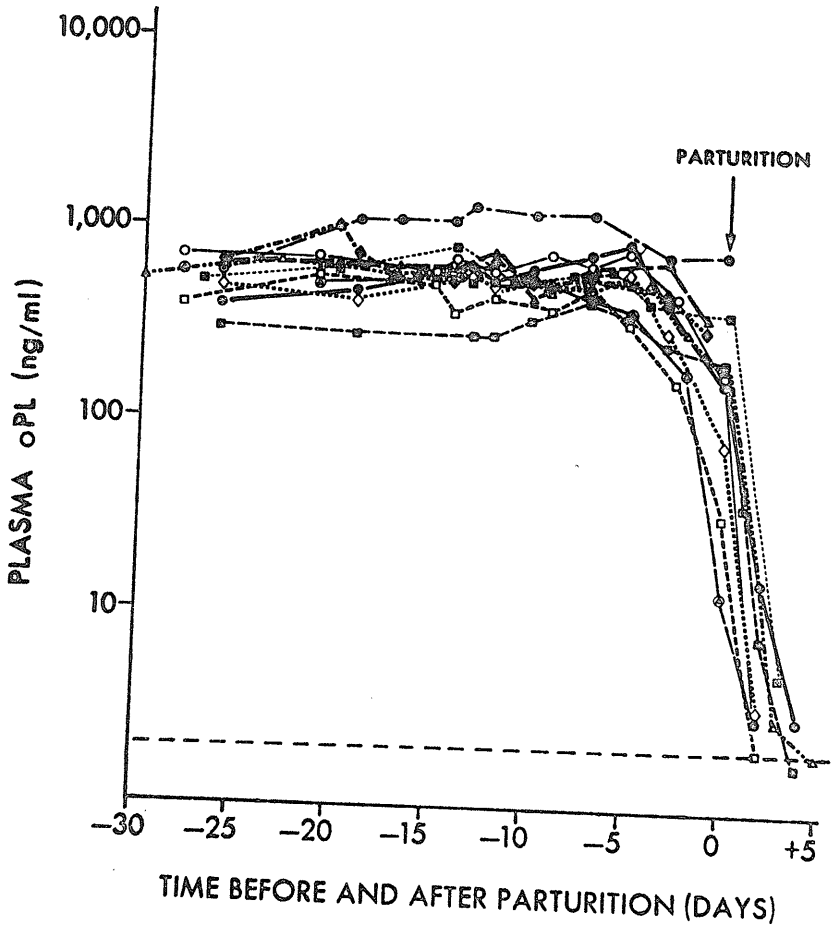

Figure 26. Plasma oPL concentrations measured by RIA during the last 20-30 days of pregnancy and 1-5 days after parturition. Eleven sheep were studied and all animals had a normal pregnancy. Each symbol represents one sheep.

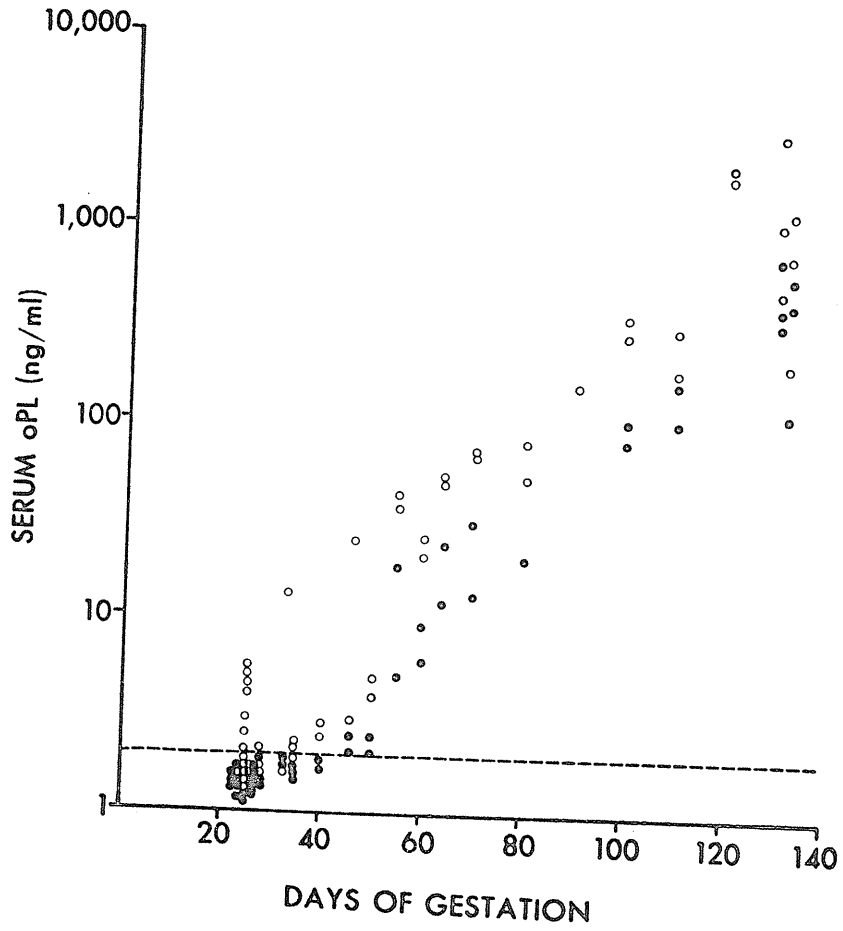

Figure 27. Uterine and jugular vein serum oPL concentrations during pregnancy, measured by RIA. Each circle represents a sample from a pregnant ewe. oPL concentration measured in peripheral blood (jugular vein blood sample), (⊙): uterine vein oPL concentration, (○).

Circadian Secretion Pattern of oPL during Pregnancy

When blood samples were taken four times daily for days from four pregnant ewes starting at approximately day 120 of gestation, it was found that there was no circadian variation in serum oPL concentrations, as shown in Figure 28.

Comparison of oPL Concentration in the Maternal and Fetal Circulation

Table IV compares oPL concentrations in maternal(peripheral) and in fetal sera at various stages of gestation. The oPL concentrations in the fetal sera were approximately nine times higher (9.5 ± 5.3; mean ± SD of five sheep) than those in the maternal peripheral sera from day 46-70, but after day 110-112, fetal concentrations were always lower than the maternal levels, amounting to approximately 20% of the maternal level.

OPL Concentrations in Allantoic and Amniotic Fluid throughout Pregnancy

Figure 29 shows oPL concentrations in allantoic and amniotic fluid at various periods of gestation. oPL was detected as early as day 18(2.5-3.5 ng/ml) in allantoic fluid and, thereafter, oPL concentrations increased to reach peak levels between day 35-50, followed by a slight decline in oPL concentrations throughout pregnancy. oPL was detected in amniotic fluid in 7 out of 9 specimens about day 40-50, but not thereafter.

Of 12 maternal urine samples collected between 110-130 days of gestation, five had undetectable levels of oPL (<2ng/ml) and the remaining seven had very low oPL levels (2-4 ng/ml).

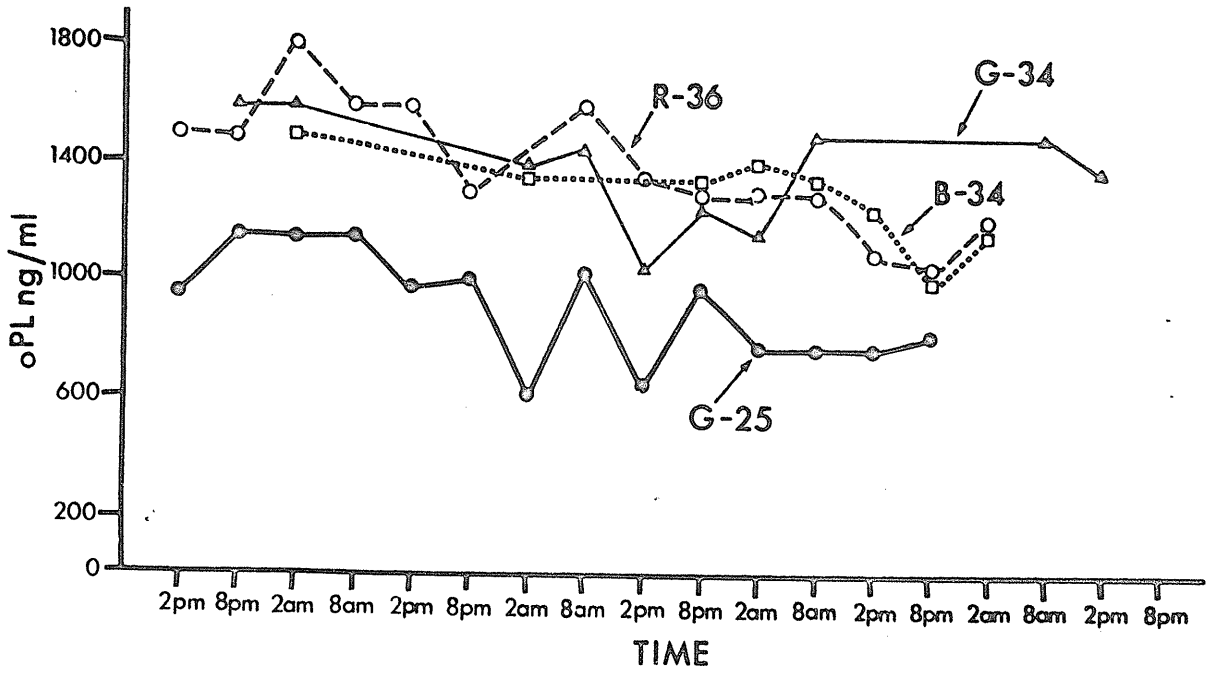

Figure 28. Serum oPL concentrations in samples obtained from four ewes at 6-h intervals over a $4\frac{1}{2}$ day period from day 120-125 of gestation.

TABLE IV

Comparison of maternal and fetal serum oPL concentrations

Sheep no.	Days of gestation	Maternal (M) oPL (ng/ml)	Fetal (F) oPL (ng/ml)				Ratio of F:M
			Fetus: 1	2	3	(Mean)	
1	46	2.5	28	48		38	15.8
2	55	18	66	75		71	3.9
3	60	9	123	123	122	123	13.7
4	64	23	95			95	4.1
5	70	13	141	132		137	10.5
6	80		78	63		70	
7	90		88	177	141	135	
8	110	175	54	51		53	0.3
9	110	700	72	49	49	81	0.08
10	110	670	70	69	94	81	0.12
11	110	190	61			61	0.32
12	110	700	67	75		71	0.10
13	110	590	77			77	0.13
14	110	160	64			64	0.4
15	110	220	59			59	0.27
16	110	290	71	44		58	0.20
17	120	410	56	95		76	0.18
18	130	375	82	68	62	71	0.19
19	130	700	60	50		55	0.08
20	130	320	50	50		50	0.16
21	132	560	40	30		35	0.06
22	132	110	24			24	0.22

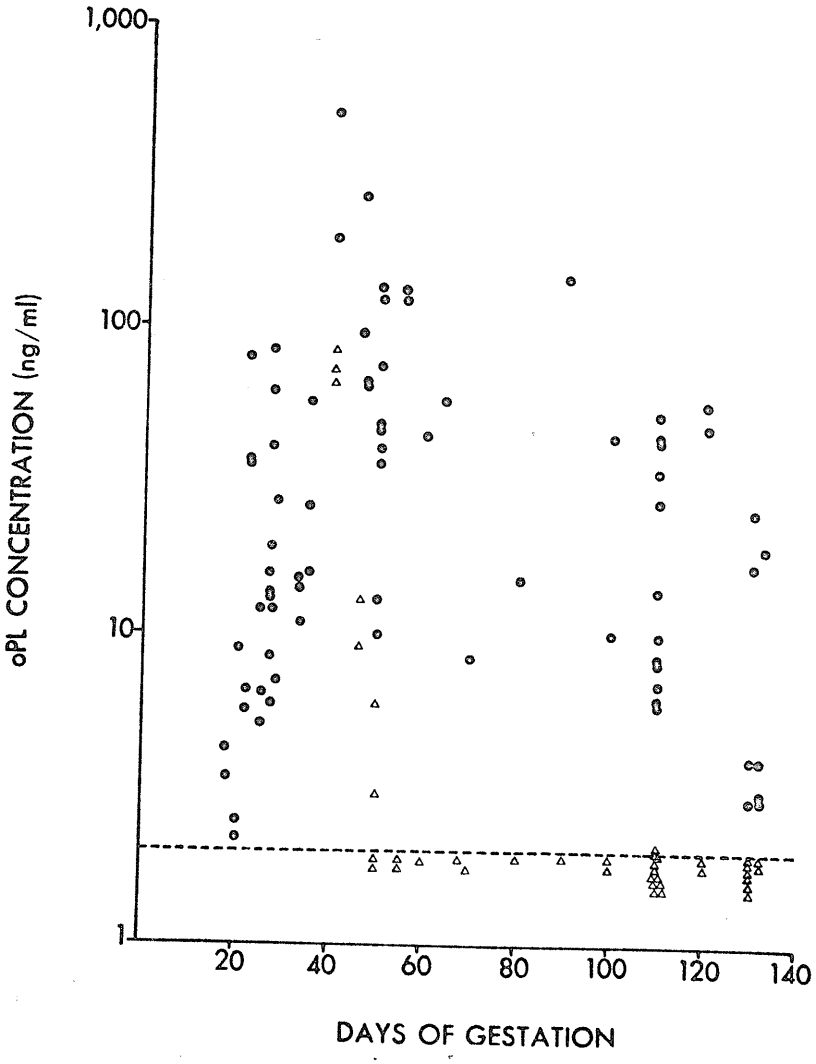

Figure 29. oPL concentrations in allantoic (o) and amniotic (Δ) fluid throughout pregnancy.

(2) Ovine placental lactogen concentration in the maternal and fetal cotyledons at various days of gestation

oPL Concentrations in Maternal Uterine Tissue Extracts

Table V shows the concentration of oPL in the extracts of maternal, fetal cotyledons, and intercaruncular tissues between 16 and 40 days of gestation. It is apparent that oPL concentrations in the extracts of fetal cotyledons were higher than in extracts of maternal caruncular tissues. Furthermore, it is also evident that oPL was also present in the intercaruncular tissues.

oPL Concentration in Fetal Membrane Extracts

Table VI shows that in the extracts of fetal membranes (chorion, allantois, and chorio-allantois), oPL concentration in chorionic extracts were higher than in the allantoic extracts before 20 days of gestation. In later gestational period, when the chorion and allantois were fused to become chorio-allantoic membrane, high concentration of oPL was found in these tissue extracts.

oPL Concentrations in PLacentomes at Various Stages of Pregnancy

Figure 30 shows the oPL concentrations in placentomes at various stages of pregnancy. In extracts obtained before day 40, oPL in fetal membranes was higher than in extracts of maternal caruncles. In placentomes, oPL concentrations reached peak levels after day 100.

TABLE V

OPL CONCENTRATION IN MATERNAL UTERINE TISSUE EXTRACTS (ng/g WET WEIGHT)

DAYS OF GESTATION	MATERNAL CARUNCLES	FETAL COTYLEDONS	INTER-CARUNCULAR
16	<10,9.6		<10,18
18	23,13,43		11,30,25
20	36,68,39,196,42,<10		40,9,43
22	96,215,71,80,74		
23	20,48		
25	74,74		
27	83,231,66		470,461,40
31	380		
33	1800,365,333	639,511,715	
35	2900,3080	20900,27300,25000	
40	10700,4500	36100,18300	

TABLE VI

OPL CONCENTRATION IN FETAL MEMBRANE EXTRACTS (ng/g WET WEIGHT)

DAYS OF GESTATION	CHORION	ALLANTOIS	CHORIO-ALLANTOIS
16	50		
18	<10,96,60		
20	<10,90,300	16.5,40	
22			281,259,183,126
23		17	
25			204
27			63,109,274,171,158 220,121,160

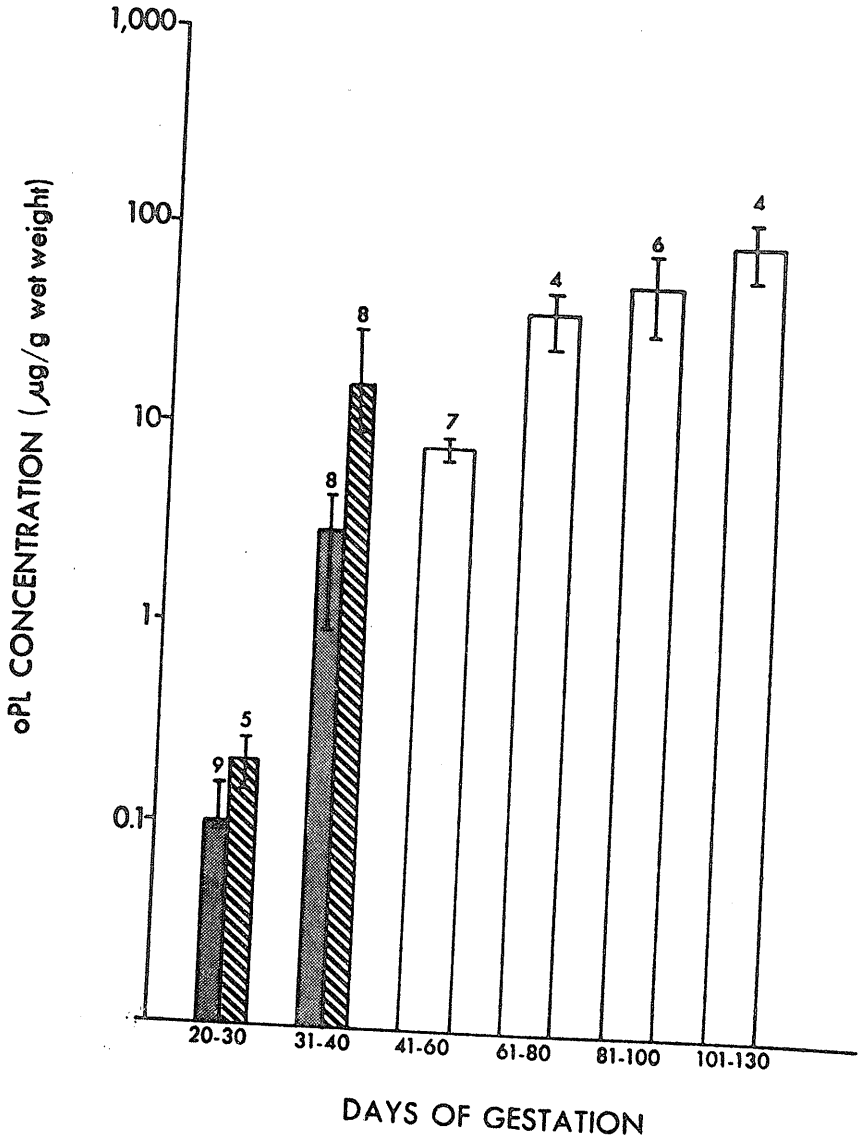

Figure 30. oPL concentrations in placentomes at various stages of pregnancy. The number above each bar indicates the number of animals studied. The vertical line indicates ± SD. Maternal caruncle extracts (shaded bars), fetal membrane extracts (hatched bars), placentome extracts (combined maternal and fetal) (open bars).

(3) Determination of Half-Time Disappearance Rate of oPL in the Sheep

1. Surgical Method

Figure 31 shows the half-time disappearance rate of oPL from maternal plasma after surgical removal of the uterus . The half-time disappearance rate of oPL calculated from the three ewes is approximately 50 to 57 minutes.

2. Injection Method

The half-time disappearance rate of radio-iodinated oPL (^{125}I-oPL) from the circulation of pregnant ewes is shown in Figure 32. A biphasic exponential curve was obtained by plotting the logarithm of the radioactivity in the TCA-precipitable material against time. Two estimates for half-time of the decay of radioactivity were denoted $t_{\frac{1}{2}}(S)$ and $t_{\frac{1}{2}}(L)$, the values of which were 1.3 to 1.7 minutes and 50 to 65 minutes respectively.

The half-time disappearance rates of oPL calculated from the samples of whole blood did not differ markedly from the values obtained from the TCA-precipitable material.

(4)Uptake of ^{125}I-oPL by Ovine Tissues in vivo

The ratio of the radioactivity found in various organs to that of blood samples taken from animals which were killed at 4 h is summarized in Table VII. At 4 h the organ to blood ratio found for all tissues remained below 1.0 with exception of kidney which has a ratio of 10 or more. This study suggests that the kidney may be degradation site for oPL in the sheep.

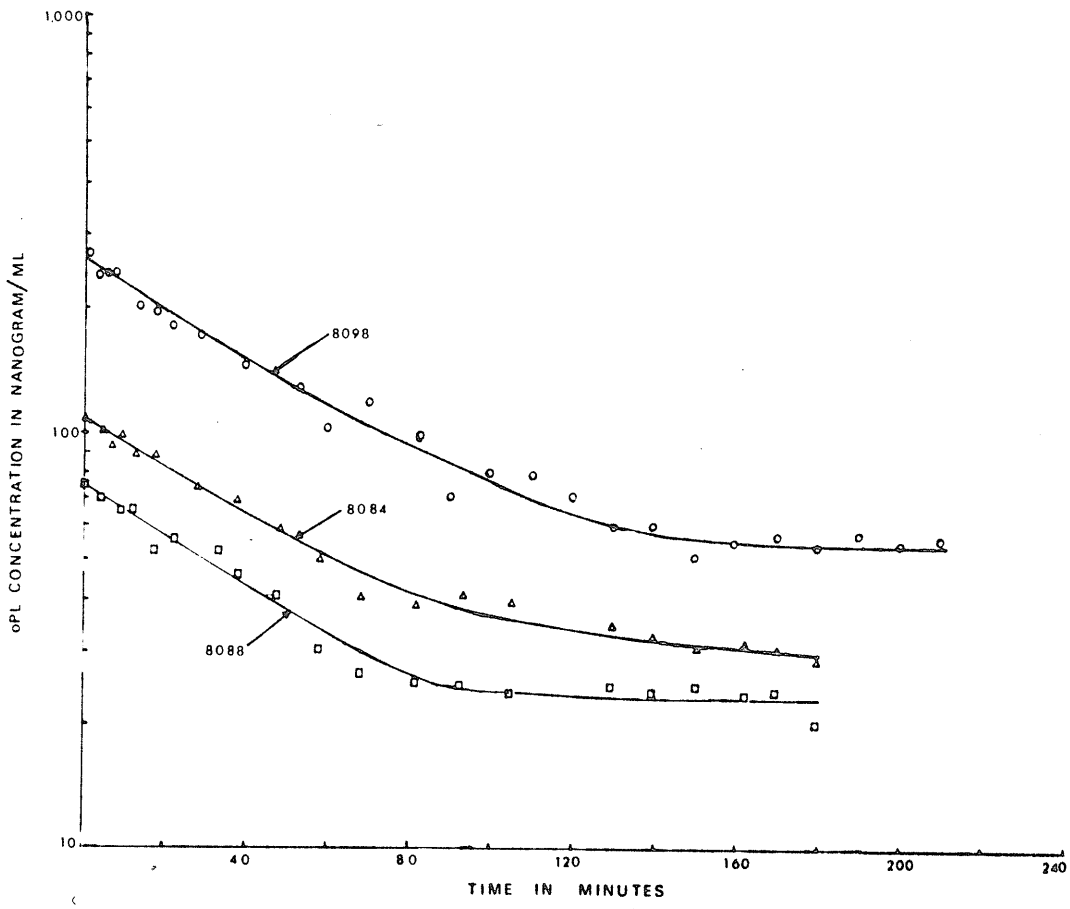

Figure 31. The disappearance of oPL following removal of the entire uterus by caesarian section in 3 pregnant ewes at 90 days of gestation. From these data it is possible to calculate that the half-time disappearance rate is approximately 50 to 57 minutes.

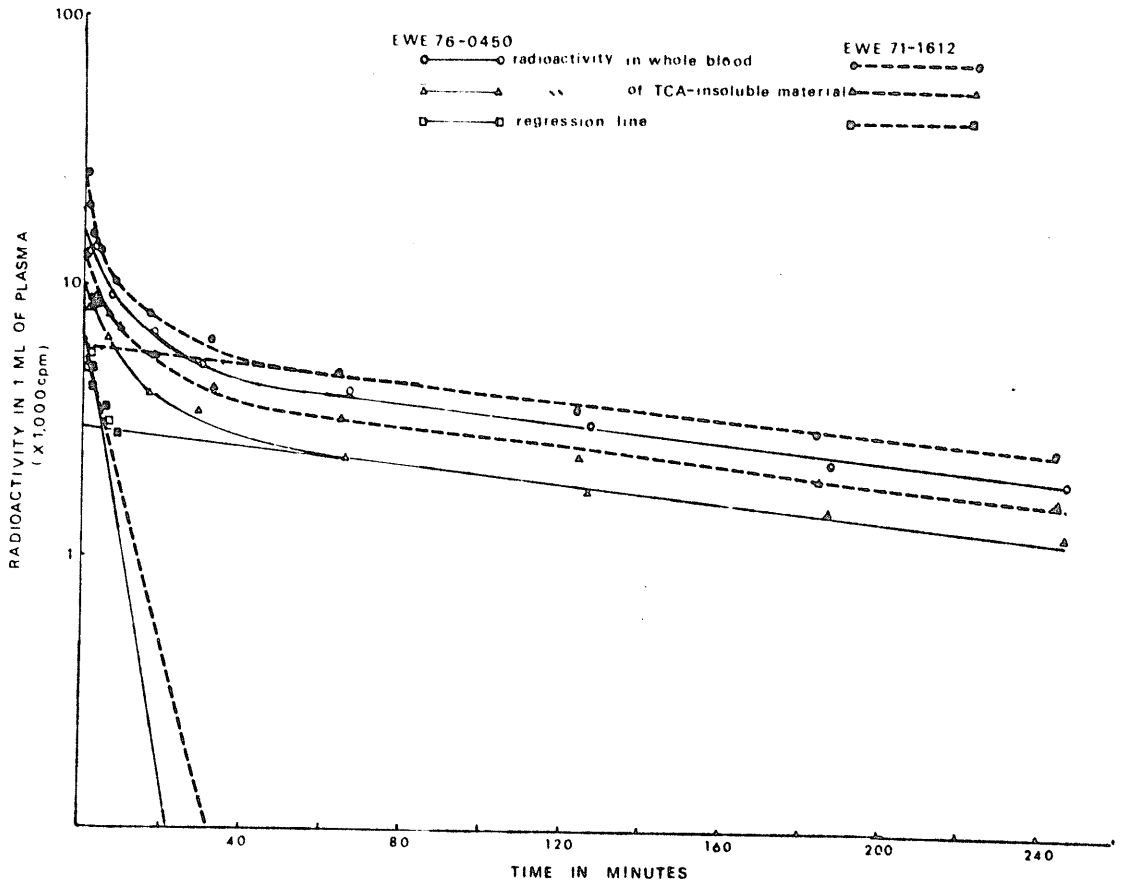

Figure 32. Semilogarithmic plot of radioactivity (○) of the trichloroacetic acid-insoluble material present in 1 ml serum of pregnant ewes at various intervals after the administration of ^{125}I-oPL. Also plotted is the radioactivity (△) in 1 ml of whole blood taken at various intervals.

TABLE VII

DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF PREGNANT EWES AT 4 HOURS AFTER INTRAVENOUS ADMINISTRATION OF ^{125}I-oPL

Tissue	EWE 1612 Organ/Blood ratio*	EWE 0540 Organ/Blood ratio
Kidney	9.68	12.7
Maternal Caruncle	0.62	0.57
Intercaruncular Tissue	0.56	0.58
Liver	0.47	0.53
Ovary(- C.L.)	0.39	0.44
Corpus Luteum(C.L.)	0.32	0.38
Adrenal	0.28	0.29
Pituitary	0.22	0.26
Muscle	0.07	0.09
Fat	0.04	0.04

* Organ/Blood ratio represents the ratio of the radioactivity in the organ (1 gram of wet weight) to the radioactivity in 1 ml of blood.

(5) Studies on the Relationships among Ovine Pituitary Growth Hormone (oGH), Prolactin(oPRL), Placental Lactogen(oPL), and Progesterone during Pregnancy in the Ewes

Homologous Radioimmunoassay for Ovine Growth Hormone

Figure 33 shows the sensivity of the homologous radioimmunoassay for ovine growth hormone. There was no cross-reaction ($<3\%$) with hormone preparations of hGH(NIH 2019, 2.2 IU/mg), mGH(NIH M945A, 0.9IU/mg), pGH(NIH P-626B,1.5IU/mg), cGH(NIH D-1001A, 1.9 IU/mg), rGH(NIH RP-1, 0.56 IU/mg), oPRL(NIH-P-S-10,26 IU/mg), bPRL(NIH-P-B_3, 18 IU/mg), oLH(NIH-LH-S-18), hPL(NIH,95% Electrophoretically pure), mPL(Dr. Shome's preparation), and oPL. The only hormone preparation which cross-reacted in the assay was bovine growth hormone (bGH,NIH B-1003A, 2.0 IU/mg).

Homologous Radioimmunoassay for Ovine Prolactin

Figure 34 shows the results of a study to determine the sensitivity and the immunological cross-reaction with hormone preparations from other species in the homologous radioimmunoassay for ovine prolactin. In this assay, bovine prolactin (bPRL, NIH-P-B_3, 18 IU/mg) showed a strong cross-reaction in the assay, whereas hGH, mGH, cGH, pGH, rGH, oLH, oTSH, oFSH, hPL, mPL, hPRL, pPRL, oPL and oGH showed no cross-reaction.

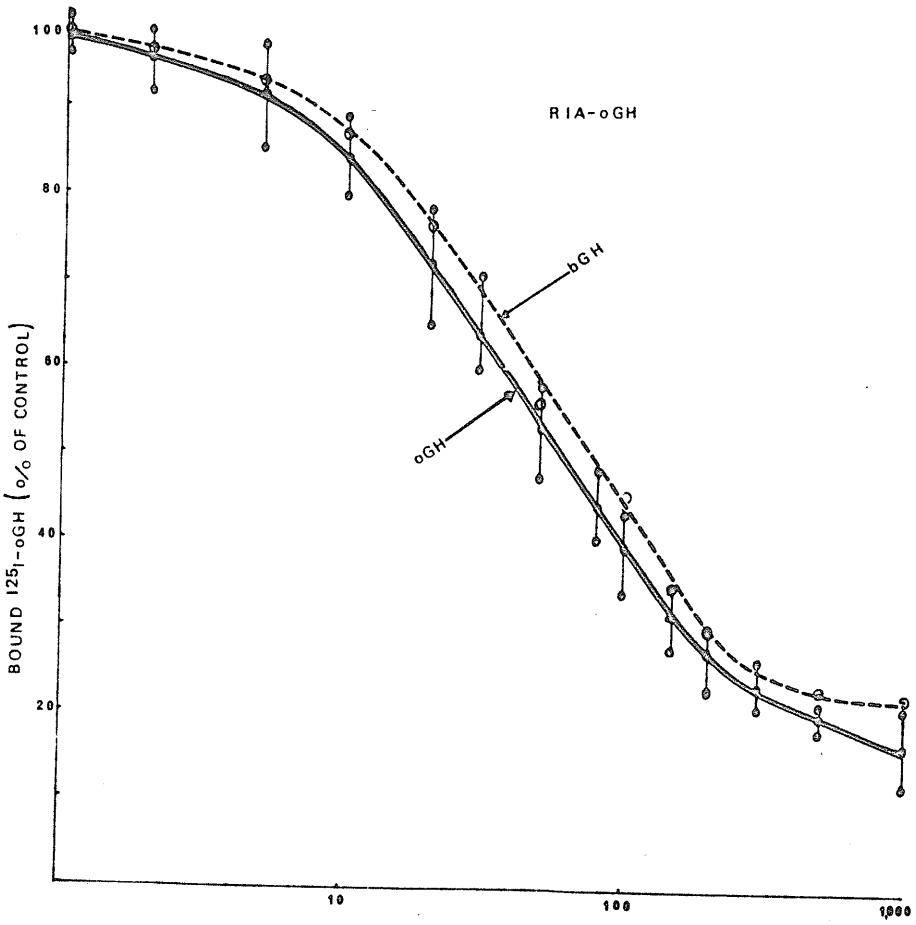

Figure 33. A radioimmunoassay for ovine growth hormone. The sensitivity of the assay is less than 10 ng/ml, oPRL and oPL as well as other ovine pituitary hormone preparations fail to inhibit the binding at concentrations less than 1000 ng/ml. Of all other growth hormone preparations from other species tested(not shown), only bovine growth hormone preparation cross-reacts in this assay. Placental extract of human, monkey, cow, sheep, goat, horse , guinea pig, rat, mouse, and rabbit do not cross-react in this assay (not shown).

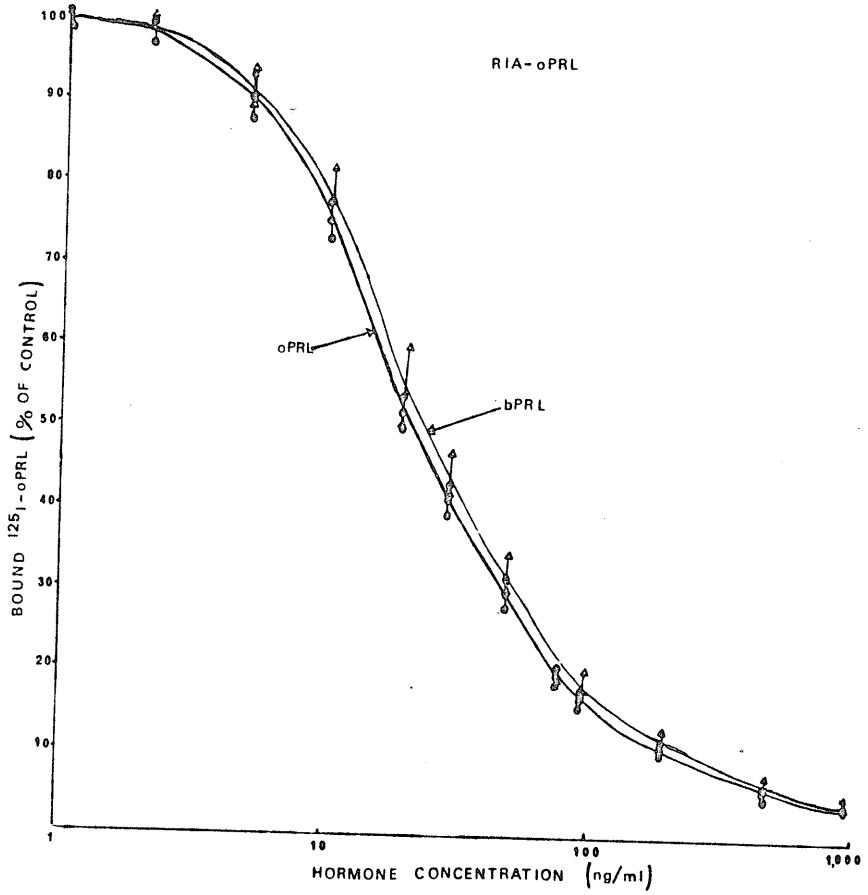

Figure 34. A homologous radioimmunoassay for ovine prolactin. The sensitivity of this assay is less than 5 ng/ml. Only bovine prolactin cross-reacts in this assay, whereas other hormone preparations do not cross -react at the concentration of less 1000 ng/ml (not shown). Similarly, placental extract of human, monkey, cow, sheep, goat, horse, guinea pig, rat, mouse, and rabbit do not cross-react in this assay (not shown).

Competitive Protein-Binding Assay for Progesterone

Figure 35 depicts a typical standard curve for progesterone in the competitive protein-binding assay using dog plasma. The lowest amount of progesterone measured by this system was 0.1 nanogram of progesterone.

Relationships among Plasma Levels of Ovine Prolactin, Growth hormone Placental Lactogen, and Progesterone during Pregnancy

The relationships between plasma levels of oPRL and oGH during pregnancy is shown in Figure 36A, whereas the relationships between oPL and progesterone concentration is shown in Figure 36B. The hormone concentrations were measured in 11 pregnant ewes at various stages of pregnancy up to 141 days of gestation. Parturition occurred at day 141-150. It is apparent that oGH levelswere not elevated significantly throughout pregnancy, and the secretion pattern of placental lactogen was similar to progesterone. Furthermore, it is evident that the concentrations of oPRL during pregnancy were inversely related to the levels of placental lactogen and progesterone.

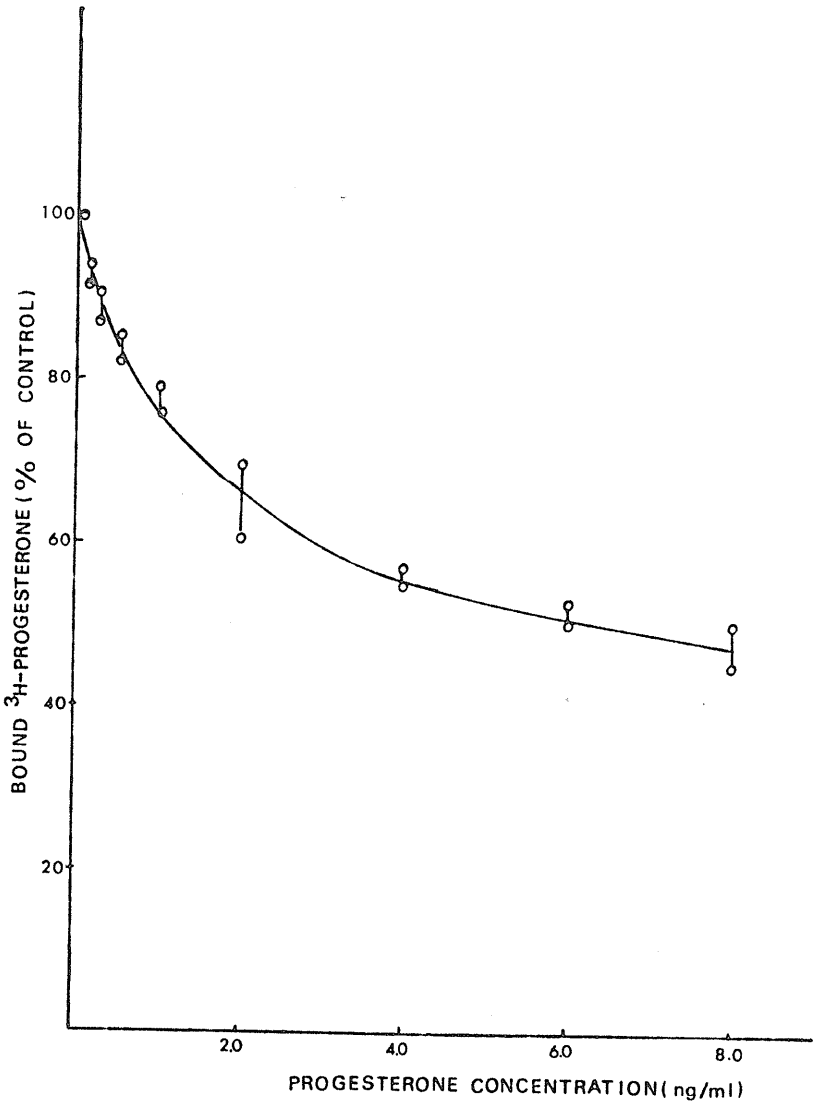

Figure 35. A typical standard curve for progesterone in the competitive protein-binding assay using dog plasma.

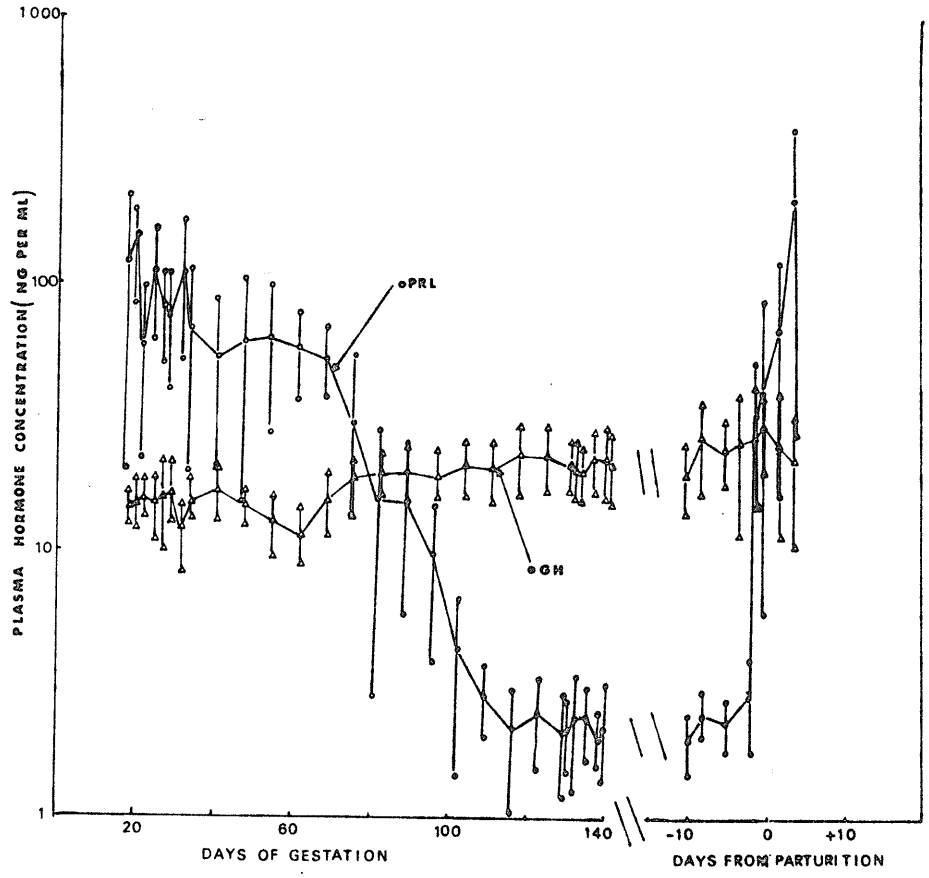

Figure 36. A. oPRL and oGH concentrations at different periods of gestation in 11 pregnant ewes. It is apparent that oGH concentration was not elevated significantly during pregnancy, whereas oPRL concentration fluctuated throughout the gestational period.

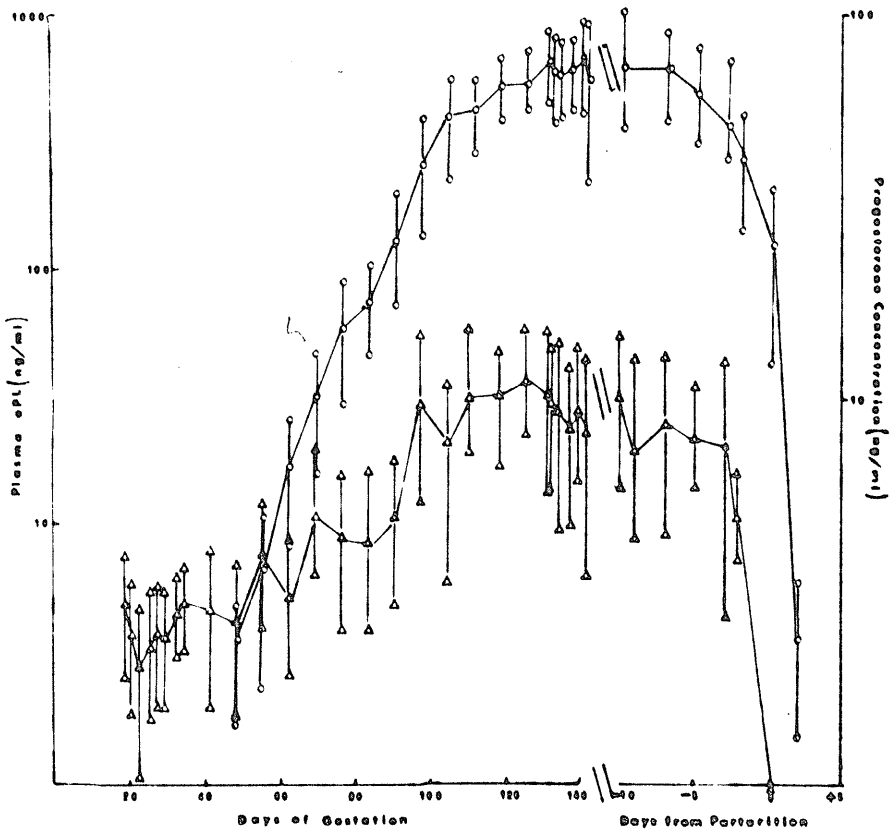

Figure 36B. oPL and progesterone concentrations at different periods of gestation in 11 pregnant ewes. The increase in oPL and progesterone is very similar for these two hormones. oPL (o--o--o). Progesterone (Δ--Δ--Δ).

E. STUDIES ON THE BIOSYNTHESIS OF OVINE PLACENTAL LACTOGEN BY THE OVINE CHORIONIC MEMBRANES in vitro

Equivalence Zone of Maximum Precipitation

Figure 37 shows the percentage of ^{125}I-oPL precipitated by 20 ul of anti-oPL in the presence of increasing amounts of unlabeled oPL, indicating that the equivalence zone of this precipitation curve is between 500 to 2000 nanogram (ng). If less than 500ng of oPL was present, incomplete precipitation occurred. When 1 microgram (ug) of unlabeled oPL was added as carrier to the reaction mixture after overnight incubation, 85-90% of ^{125}I-oPL was precipitated , provided that the amount of oPL in the initial solution did not exceed 2 ug. In subsequent precipitation studies of ^{3}H-oPL, we first determined the total amount of oPL by RIA, and then added carrier oPL into the incubation medium ensuring that the oPL amount did not exceed 2 ug.

Biosynthesis of ^{3}H-proteins and ^{3}H-oPL by Chorionic Membranes of Various Gestational Period

Table VIII shows the amount of radioactivity which could be precipitated by TCA and anti-oPL in various incubation media. It is apparent from this table that ovine chorionic membranes were able to synthesize and to secrete oPL as early as 26 days of the gestational period.

Gel Filtration Pattern of Incubation Medium on Sephadex G-100

The distribution of protein and oPL when an aliquot (3 ml) of 24 h incubation medium was fractionated on a Sephadex G-100

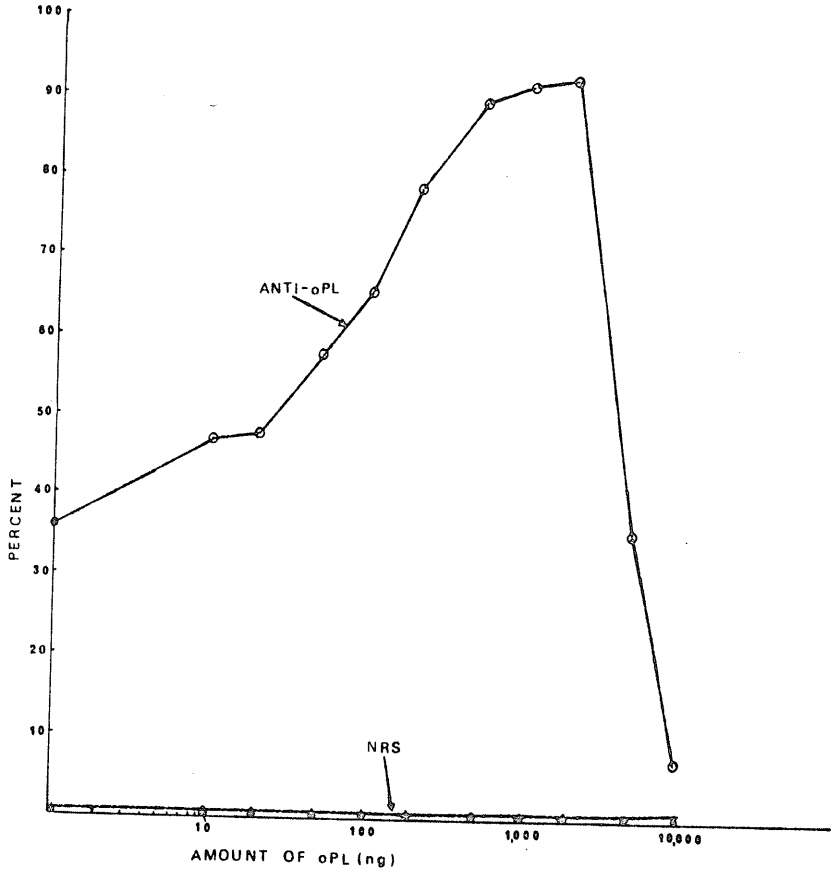

Figure 37. Percentage of ^{125}I-oPL precipitated by anti-oPL in the presence of increasing amounts of unlabeled oPL. Normal rabbit serum(NRS).

TABLE VIII

BIOSYNTHESIS OF OVINE PLACENTAL LACTOGEN BY CHORIONIC MEMBRANES OF EARLY PREGNANT EWES in vitro

Days of Gestation	Total RIA-oPL in Medium (nanograms in 5 ml)	^{3}H-Proteins(TCA-Ppt) (X 10^{5} cpm in 5 ml)	% of Specific Count* (% ^{3}H-oPL)
26	90	2.16	2.29
26	140	2.93	2.89
26	120	2.58	4.87
26	105	2.75	1.69
32	73	2.80	1.54
32	110	2.23	2.10
32	105	4.73	2.43
32	113	5.95	4.17
58	120	2.48	5.76
58	150	2.75	5.61
58	115	2.90	3.90
58	135	2.63	6.52

*% of Specific Count is calculated as follows :(Anti-oPL Ppt ^{3}H-Count – NRS Ppt ^{3}H-Count)/ TCA Ppt ^{3}H-Count X 100 %

column is shown in Figure 38A. Similarly, the distribution of radioactivity was shown in Figure 38B. Two major radioactive peaks emerged. The first peak (elution volume 30-45 ml) is associated with proteins of molecular weight greater than 100,000. The second emerged in the elution volume (130-150 ml) corresponding to the small molecular weight substances, including free ^{3}H-leucine, which were soluble in TCA. However, when the radioactive proteins were precipitated by anti-oPL, two peaks of radioactivity are noted. The first peak of radioactivity corresponds to the molecular weight of greater than 100,000 (the void volume), and the second peak of radioactivity corresponds to proteins of molecular weight of 20,000 to 25,000 as calculated from the elution volume.

Furthermore, it is apparent that the amount of radioactive proteins that could be precipitated by anti-oPL was about 2-3 % of the total TCA-precipitable radioactive proteins in the first peak. In the second peak, about 10% of the TCA-precipitable radioactive proteins could be precipitated by anti-oPL.

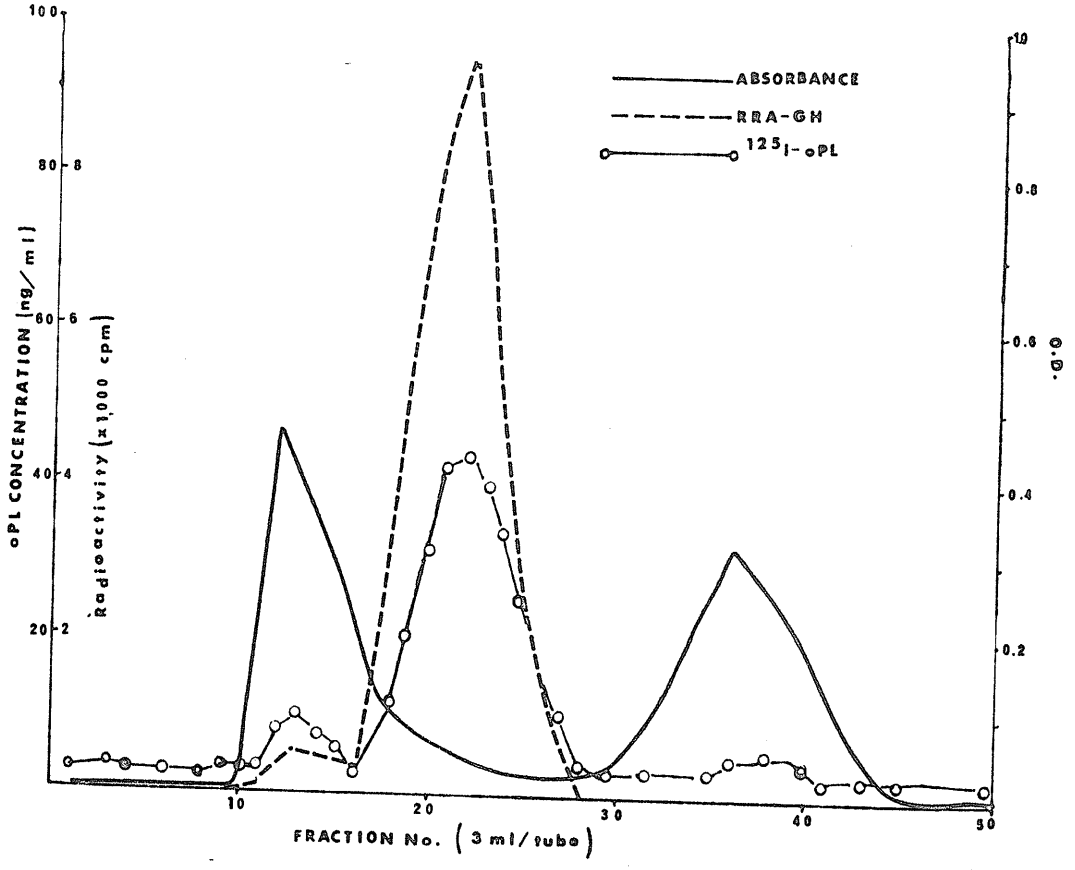

Figure 38A. Distribution of protein and oPL after gel filtration of incubation medium (24 hr) on Sephadex G-100.

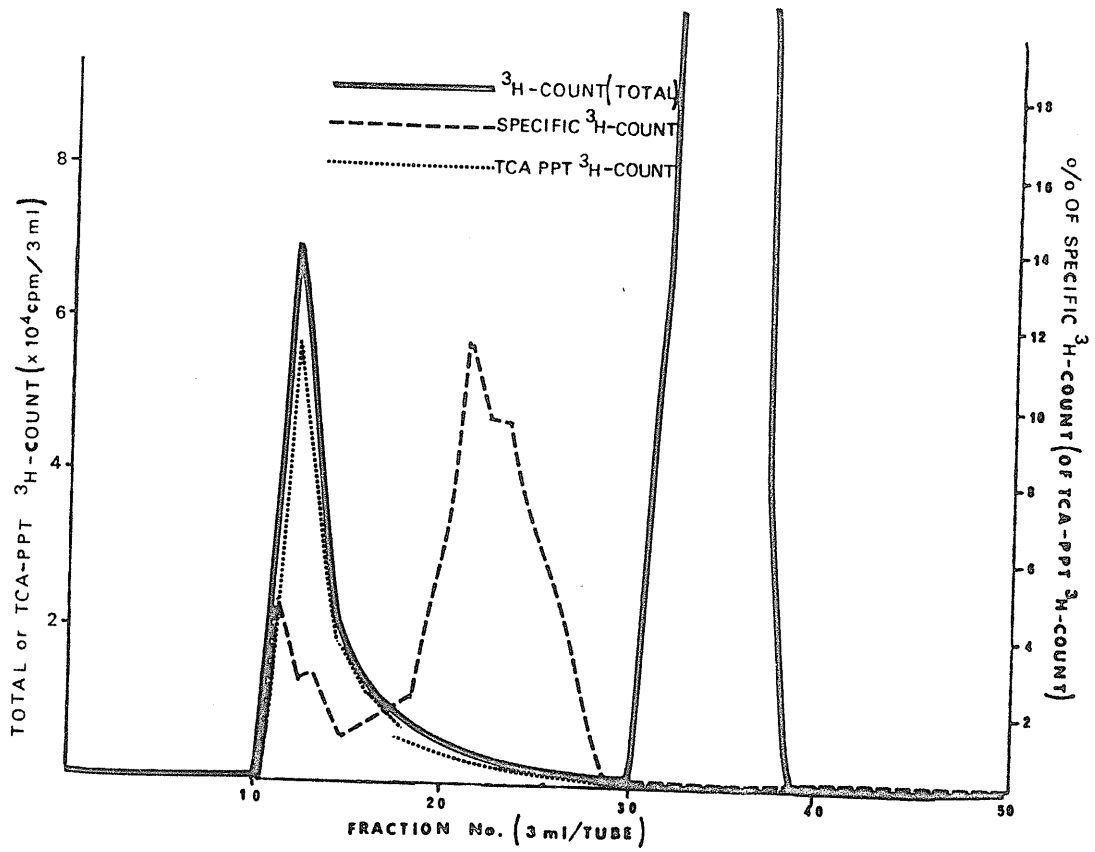

Figure 38B. Distribution of radioactivity after gel filtration of incubation medium (24 hr) on Sephadex G-100. Two proteins of widely differing molecular weight were precipitated by anti-oPL.

F. STUDIES ON THE BIOLOGICAL EFFECTS OF OVINE PLACENTAL LACTOGEN

(a) Growth Promoting Effect of oPL in Hypophysectomized Rats

Table IX shows the results of the bioassay for growth promoting activity of the partially purified oPL. This assay demonstrated that oPL is a potent growth promoting hormone in stimulating the growth of epiphyseal plate of tibiae of hypophysectomized rats.

(b) Luteotropic Effect of Ovine Placental Lactogen

Radioimmunoassay for Progesterone

Figure 39 shows a typical standard curve using the radioimmunoassay for progesterone. The sensitivity of the assay is less than 25 picogram (pg) of progesterone.

Radioreceptorassay for Luteinizing Hormone-like activity(RRA-LH)

A typical standard curve of hCG in the radioreceptorassay for LH is shown in Figure 40. In this assay, ^{125}I-hCG, hCG, and pseudopregnant rat corpora lutea homogenate were used as tracer, hormone standard, and receptors respectively.

Effects of Prostaglandins, Prolactin, oPL, and hPL on Gonadotropin Receptors of the Corpus Luteum and on Serum Progesterone.

Figure 41 shows that within 24 hours PgF_{2a} markedly decreased the binding capacity of C.L.by about 50% and serum progesterone

TABLE IX

BIOASSAY OF GROWTH PROMOTING ACTIVITY IN HYPOPHYSECTOMIZED RATS

Treatment Total Dose/Rat		Body Weight(gm) Initial	Final	Ovarian Weight (mg)	Adrenal Weight (mg)	Epiphyseal Width(Micra)
Controls BSA Saline	Av.	82	83	7.3	14.6	141.4
	S.E.±			0.35	0.68	5.03
Int. Std. bGH 0.025 i.u.(25ug)		81	85	8.1	16.2	191.5**
				0.30	0.36	6.35
Int. Std. bGH 0.075 i.u.(75ug)		81	87	8.1	18.2**	219.5**
				0.27	0.61	2.75
Int. Std. bGH 0.225 i.u.(225ug)		82	84	8.3*	17.6**	236.3**
				0.23	0.51	8.40
# 21(D) 20 ug		82	86	6.6	17.3**	192.5**
				0.28	0.69	11.62
# 21(D) 40 ug		81	85	6.4	17.7**	196.7**
				0.29	0.55	10.12
# 21(D) 80 ug		83	88	6.4	16.3	227.2**
				0.64	0.75	10.36

* Significantly different from controls ($P < 0.05$)

** Significantly different from controls ($P < 0.01$).

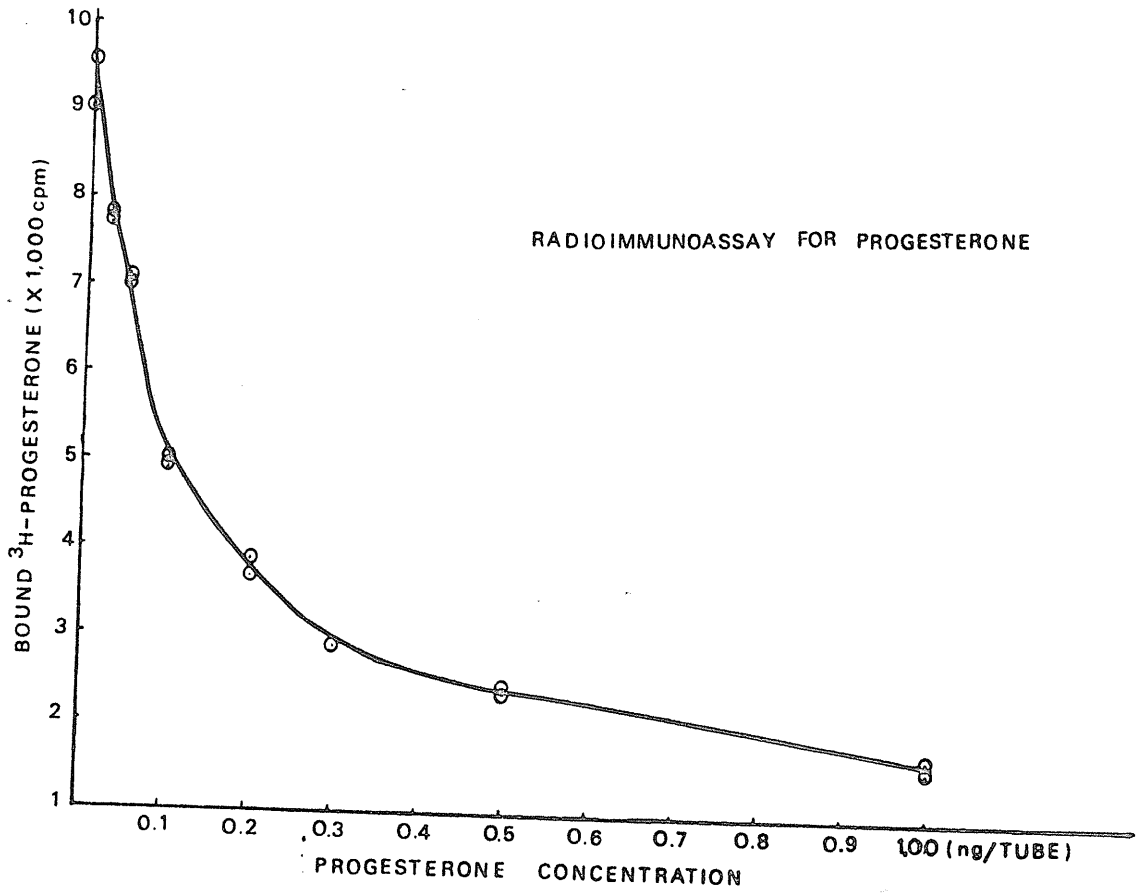

Figure 39. Typical standard curve of progesterone in the radioimmunoassay for progesterone.

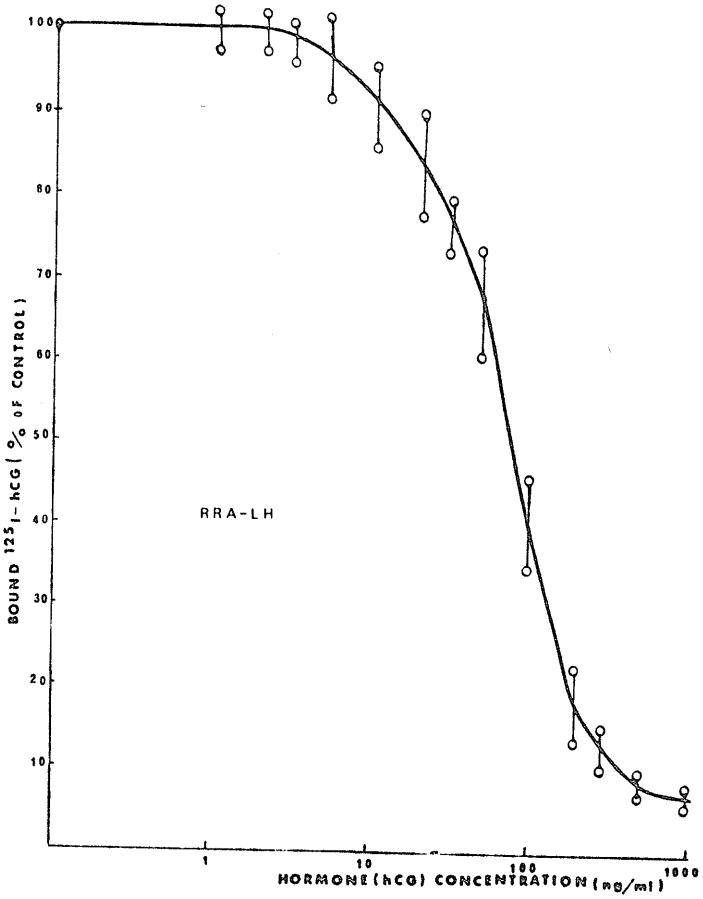

Figure 40. Radioreceptor assay for LH using ^{125}I-hCG, hCG, and pseudopregnant rat corpora lutea as tracer, hormone standard, and receptor respectively.

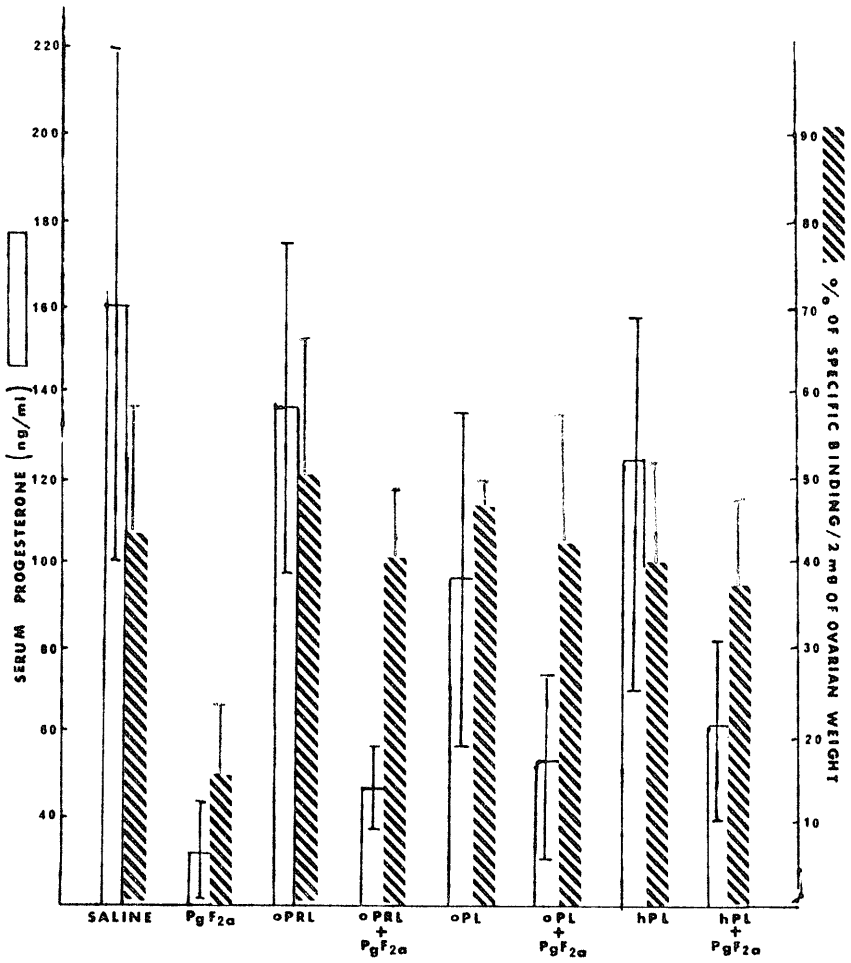

Figure 41. Effect of PGF_{2a} and lactogenic hormones on LH receptor and serum progesterone. Mean ± SD.

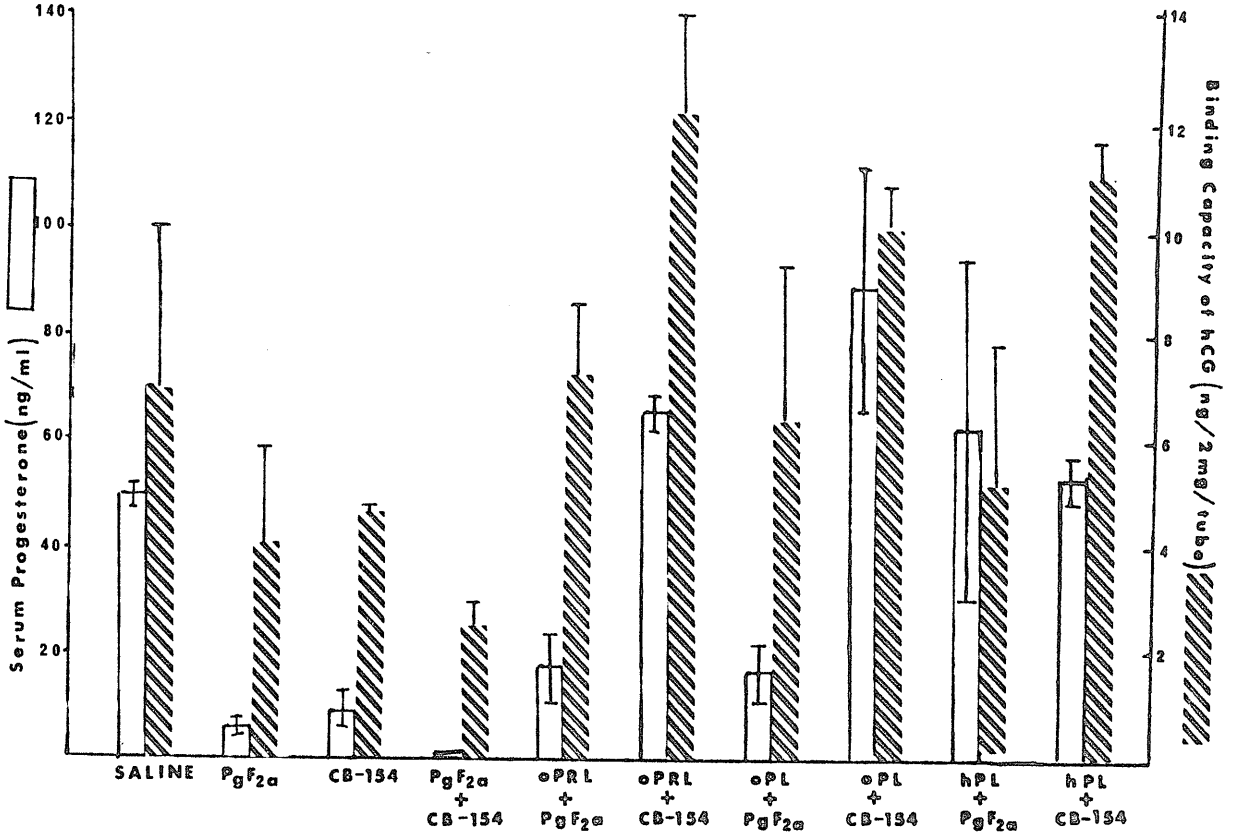

Figure 42. Effect of PGF_{2a}, ergocryptine, and lactogenic hormones on LH receptor and serum progesterone. Mean±SD.

by 85%. In this experiment, oPL, oPRL, and hPL did not increase the specific binding activity of hCG to pseudopregnant corpora lutea. However, pre-treatment of the animals with either oPRL, oPL, or hPL before PgF_{2a} administration prevented the drastic decrease of gonadotropin binding activity and the drop in serum progesterone produced by PgF_{2a} alone. This study suggests that lactogenic hormones, particularly placental lactogens, can act like PRL in maintaining the LH-receptors in the corpus luteum.

In order to provide evidence of a possible interaction of exogeneous lactogens on the LH-receptors in the rat corpora lutea, animals were administered with ergocryptine (CB154), a drug known to depress prolactin secretion in the rat (Shau and Clemens, 1972). Figure 42 demonstrated that lactogens were completely effective in blocking the loss of LH-receptors and the decrease of serum progesterone caused by CB-154. Thus, this study demonstrated that placental lactogen can maintain the corpus luteum enhancing its response to LH and may indicate the mechanism of interaction of these hormones in the control of corpus luteum function during pregnancy.

SECTION VI : DISCUSSION

A. PURIFICATION AND CHARACTERIZATION OF OVINE PLACENTAL LACTOGEN

Assay to monitor the hormonal activity of oPL throughout purification

The reasons that I employed only RRA-GH to monitor the hormonal activity of ovine placental lactogen during purification were several : 1. We had previously shown that growth hormone-like and prolactin-like activities resided in the molecule of oPL (Chan et al,1976); 2. The reproducibility of the RRA-GH is higher than RRA-PRL(90% compared to 65% reproducibility); 3. ^{125}I-hGH is more stable than ^{125}I-oPRL during storage at -20 C. and 4. The availability of rabbit liver in our laboratory is better than the availability of rabbit mammary gland.

Extraction and Purification

In our initial attempts to purify oPL from frozen placental cotyledons, various difficulties were encountered. In the primary extraction procedure, the alkaline extract was turbid and viscous and could not readily be cleared by centrifugation or filtration. The viscosity of early-term placental extracts (54-65 days) was greater than that of extracts obtained from near term (130 -145 days)

or placental extracts obtained post partum. The nature of the factors contributing to the viscosity is not clear, but mucous substances, such as mucin and mucopolysaccharides, are suspected. When this thick extract was directly applied to a DEAE-cellose column poor resolution and blockage of the column were obtained unless small batches of placental cotyledons were used. The fractional precipitation with lower pH (pH 6.5) proved to be a valuable step in the purification of oPL. This procedure precipitated a lot of mucous substances which were responsible for the blockage of the DEAE-column.

It is apparent that CM-Sephadex C-50 column chromatography provide superior resolution than CM-cellulose column chromatography. Similarly, the combined property of molecular sieving and ion-exchange in DE-Sephadex A-25 gave better superior resolution than DEAE-cellose. However, one of the disadvantages in employing CM-Sephadex is that the flow rate is slow. Moreover, it is desirable to use a relatively small column or else extremely slow flow rates or even blockage of the column will occur.

In summary, by employing these procedures I am able to purify ovine placental lactogen from ovine placental cotyledons greater than 2,000 fold. The percentage of recovery is greater than 5%.

Characterization

Analytical polyacrylamide gel electrophoresis-

In acidic polyacrylamide gel electrophoresis (pH 4.3-4.5), only one stained band was seen. The presence of oPL was detected in eluants from segments which corresponded to the stained band. Thus, the data from acid gel suggested that the oPL preparation is homogeneous. In alkaline polyacrylamide gel electrophoresis (pH 9.3-9.5), three stained bands were seen which were not well separated. The distance between each band being approximately 0.2-0.3 cm apart. When the gel was divided into segments, and when the segments were eluted and oPL activity determined by the RRA-GH, some overlap in activity of the two darkest bands was apparent. The reason for this observation is not clear at present. However,one possible explanation for this observation may be that aggregation of the hormone occurs in alkaline buffer. Studies of bGH by Dellacha et al (1968), hPL by Belleville et al (1975), bPL by Bolander et al (1976), and oPL by Hurley et al (1977) all have shown aggregation of the hormones. Hurley et al (1977) have reported that oPL aggregates reversibly in low ionic strength alkaline buffer and at high protein concentrations. The exact amount of contaminating proteins (the least stained band) in our oPL preparation is uncertain, since the intensity of the stained band may not

be proportional to the amount of protein present. However, if we assume that the intensity of the stained band is proportional to the amount of protein present and is similar for all proteins than I could conclude that oPL constituted more than 80% of the protein in the preparation.

Analytical gel isoelectric focusing-

In the present studies, we employed cytochrome C as an indicator and assumed that a steady state gradient was set up for oPL when cytochrome C passed over the cathode. Whether this was the time that a steady-state had been established remains uncertain. Again, by employing this sensitive technique, 3 stained bands were separated by at least 0.3 cm. The presence of GH-like activity wasdetectedin eluants from segments which corresponded to the two stained bands. Some overlap in activity of the least stained band was also apparent.

Estimation of molecular weight by polyacrylamide gel (slab) containing sodium dodecyl sulfate (SDS)-

Comparison of the mobilities of oPL with other known molecular weight proteins in polyacrylamide gels containing SDS indicate that oPL, hPL, and oPRL are of similar

size, corresponding to the molecular weights of 19,500 to 21,500. Again a small amount of contaminating proteins were apparent by this technique. Two bands were seen in the gel, the minor one is approximately 10% of the major band based on the intensity of the stain.

In summary, analysis of the oPL preparation upon electrophoresis revealed that it was not homogeneous. The purity of the oPL preparation is estimated to be approximately 80-90%. Molecular weight estimation of oPL is in the range of 19,500 to 21,500.

Displacement curve of oPL in the radioreceptorassay for growth hormone (RRA-GH) using rabbit tissue-

In this assay, we employed ^{125}I-oGH as tracer and standard respectively. The reason that we did this is that oGH exhibits only somatotropic and no lactogenic activity, whereas hGH produces both activities in the receptor assays (Shiu et al,1973 and Tsushima et al,1973). Therefore, by using ^{125}I-oGH and oGH as tracer and standard respectively, we are more confident of the estimate of growth hormone-like potency of oPL.

Binding of ovine placental lactogen to human liver (100,000 x g fractions)- Only oPL and hGH react equally in the two receptor assays, that is, have equal growth hormone-like and lactogenic activities. This observation hinted at the possibility that oPL and hGH might also have certain common structural features, and suggested that it would be worthwhile examining the binding of oPL to a human tissue receptor for hGH. Lesniak et al (1973) have reported that human lymphocyte receptor for growth hormone is species-specific, and that non-primate growth hormones fail to compete for the binding sites. These same features were observed by Carr et al (1976) using a human liver receptor assay. It was therefore of great interest to find that oPL binds very effectively to human liver, whereas hPL minimally and non-primate growth hormone preparations not at all. These observations indicate that oPL has biological and perhaps structural and conformational features very similar to those of hGH.

B. DETECTION AND CHARACTERIZATION OF RECEPTORS FOR OVINE PLACENTAL LACTOGEN IN THE SHEEP

Distribution of Binding of Ovine Placental Lactogen to Various Tissues

The highest specific binding of oPL was observed in the 100,000 x g pellet of several tissues (liver, adipose tissue, ovary, corpus luteum, non-pregnant uterus, and fetal liver). In all cases,

the specific binding of oPL to 780 x g and 15,000 xg pellets was less than 50% of the binding associated with the 100,000 x g pellet. There seemed to be very little difference in oPL binding to tissues obtained from non-pregnant, early pregnant(22-27 days gestation), or late pregnant (130-135 days gestation) ewes, with the exception of adipose tissue and uterus. In the latter two, binding of oPL decreased approximately 50% in late gestation when compared with the non-pregnant period. The exact reason for this decrease is not clear.

The low binding of oPL to mammary gland 100,000 x g fractions (less than 3%) was unexpected. A potent PRL-like effect of oPL has been demonstrated in the rabbit (Chan et al,1976; Handwerger et al ,1974), in the pigeon crop sac bioassay and receptor assays, and mouse mammary gland explants (private communications with Drs. Charles Nicoll, Isabel Forsyth, and Fred Leung, respectively). In binding studies one must always remain concerned that inappropriate conditions have led to a negative result rather than that there is any absence of binding sites.

Therefore, several attempts were made to optimize conditions for oPL-binding studies using mammary gland tissue preparations including variations in temperature, time of incubation, pH and buffer systems, and tissues at different gestational periods. In all cases, less than 3% specific binding per milligram of protein was obtained. If we assume that our studies are valid and that there are few oPL sites in the mammary gland, questions arise

whether oPL is mammotropic in the ewe, despite the fact that earlier experiments performed by other investigators have suggested that the sheep placenta may secrete a mammotropic substance(Denamur et al,1961).

Properties of oPL-receptors

Effect of protein, time and temperature on the binding of oPL to ovine liver, adipose tissue, ovary, corpus luteum, non-pregnant uterus, and fetal liver- Like the binding of oPRL to rabbit mammary gland (100,000 x g fractions)(Shiu et al,1974), the binding of oPL to its receptors are protein, time and temperature dependent. At 37 C, binding of oPL to the liver, adipose tissue, corpus luteum, nonpregnant uterus, and fetal liver reaches its maximum within 1 hr. In the case of the ovary, the maximal binding of oPL is not reached until after 24 hr incubation at 37 C. At the present, we have no good explanations for this discrepancy.

Specificity of oPL-receptors- It is interesting that in the radio-receptor assay for oPL using either ovine liver, adipose tissue, ovary, corpus luteum, uterus, or fetal liver 100,000 x g fractions, only oGH inhibited the binding of ^{125}I-oPL in a parallel manner but exhibited only 1/2 to 1/5 the potency of oPL, whereas oPRL and oLH failed to inhibit. These findings suggest that, functionally, the activity of oPL may be more closely linked with GH-like actions

than with prolactin-like effects. The growth-promoting activity of oPL has been demonstrated convincingly in hypophysectomized rats(Chan et al,1976), in which it not only promotes body weight but also stimulates growth of the tibial cartilage as shown in Table IX. But what GH-like effects of oPL might be observed during pregnancy might be ascribed to oPL ? By analogy to hPL, one might expect oPL to promote release from adipose tissues of free fatty acids to be used by the maternal tissues (a glucose-sparing effect) to ensure a steady source of available energy for the fetus, since glucose is the main substrate for the fetus in the ewe(Leat,1971). Thus, the binding of oPL to adipose tissues is of interest. However, recent reports from Handwerger et al (1975;1976) indicate that injection of oPL into ewes (both pregnant and non-pregnant) caused a significant decrease in plasma concentrations of free fatty acid, suggesting that oPL may be anti-lipolytic in vivo. Direct confirmation of this anti-lipolytic effect of oPL on sheep adipose tissue in vitro would be welcome. In the rhesus monkey, oPL also seemed not to be lipolytic (private communications with Dr. Julane Hotchkiss, University of Pittsburg).

The fact that oPL binds to 100,000 x g pellets obtained from sheep ovaries and corpus luteum is of considerable interest. This is especially true because in the case of the ovary, both oLH and oPRL slightly inhibited the binding of oPL in a non-parallel manner whereas in other tissues they do not. The non-parallel inhibition exhibited by oPRL and oLH are difficult to interpret. One possible

explanation is that there are receptors in the ovary which are able to bind the specific common sequence of ovine placental lactogen and oPRL but the affinity for these two hormones are substantially different, hence the inhibition curves are different.

The present results differ in some respects from those of Bolander et al (1976). In their report, oPL bound to pregnant rabbit adrenal, liver, ovary, mammary gland, uterus, kidney, brain, and fat, and oPRL displaced ^{125}I-oPL in a parallel manner. In our studies, significant binding of oPL to adrenal, mammary gland, kidney, and brain 100,000 x g microsomal fractions was not observed, and oPRL failed to displace ^{125}I-oPL in a parallel manner in liver, adipose tissue, ovary, uterus, and fetal liver. These differences stress the importance of species differences that have been noted in hormone binding studies.

Binding of oPRL to Sheep Tissues

Ovine prolactin is active in promoting casein synthesis and mammary gland growth in experimental animals (Handwerger et al, 1974; Chan et al,1976), and yet we could not demonstrate greater than 3% specific binding in ovine mammary glands as well as other ovine tissues studied. Indeed the only tissue to exhibit considerable binding of oPRL is the uterus of the pregnant ewe in which the specific binding increased from less than 3% to more than 14%/mg protein near term. At the present stage, one can only speculate that

the increase in oPRL binding sites might be related to parturition as in other species it has been postulated that PRL may influence uterine motility(Manku et al,1973).

At present, we are puzzled about the failure of oPRL as well as oPL to bind to ovine mammary tissues. Both uterus and mammary tissues were handled in the same manner; in the former, binding sites for PRL were readily demonstrable (40 fmol/mg protein) but in the latter none could be identified. These data suggest that the methodology for identifying PRL binding sites in sheep tissues probably are adequate unless one wishes to postulate a unique degrading system for these sites in the mammary gland. No such evidence was obtained when equal amounts of uterine and mammary membranes were used together in binding studies.

Comparative Studies of PLacental lactogen-like, Growth hormone-like, and Prolactin-like activities in Placental Extracts of Several Species

Factors with growth hormone-like and prolactin-like activities have been quantitated in a number of species using RRA-GH and RRA-PRL by Kelly et al(1976). In their studies, Kelly et al utilized hGH and oPRL as standards in the RRA-GH and RRA-PRL respectively. The purpose of our studies is to confirm the presence of placental lactogens in species which have previously been identified as having placental lactogen by employing homologous radioreceptor assay for placental lactogen, that is, a radioreceptor assay using

sheep liver as receptor and sheep hormone(oPL) as standard.

In the present study we have identified and quantitated factors with placental lactogen-like activity not only in the placental extracts of primates (human and monkey) and ruminants (sheep, goat) but also in the mouse and guinea pig. To our surprise, we could not detect PL-like activity in the placental extracts of cow, dog, rat, horse and rabbit. In contrast, prolactin-like activity has been detected in the placental extracts of human, monkey, sheep, goat, mouse, guinea pig, cow, dog, rat, but not in horse and rabbit by the RRA-PRL. By employing RRA-GH, GH-like activity has been detected in the placental extracts of primates and ruminants. At the present, we are puzzled at the discrepancy among the results obtained from RRA-PRL and RRA-PL. It is not clear why lactogenic activity could be detected in the placental extracts of cow, dog, and rat by RRA-PRL but not by RRA-PL. One of the possible explanations could be that structurally the molecules of placental lactogen of cow, dog, and rat are more similar to oPRL than to oPL. Clearly, more definitive conclusions will be obtained only following the purification of the respective lactogens.

C. RADIOIMMUNOASSAY FOR OVINE PLACENTAL LACTOGEN

The results obtained indicate that oPL is immunochemically different from sheep pituitary hormones, i.e. GH and PRL as well as from placental lactogens of several other species. This difference may account for the past failure to detect ovine or other placental lactogens by using RIAs for oGH and oPRL(as shown in Figure 33 and 34). However, Handwerger et al (1974) reported that an antiserum to oPL partially cross-reacted with oGH by using immunodiffusion techniques. It is possible that some antisera may exhibit different specificities or it may be that there are differences between Handwerger's oPL preparation and ours.

D. STUDIES ON THE SECRETION OF OVINE PLACENTAL LACTOGEN

1. Maternal and Fetal Secretion of Ovine Placental Lactogen

By radioimmunoassay, oPL is detectable as early as the 48th day of gestation in maternal peripheral plasma and the 25th day of gestation in maternal uterine vein sera of some ewes, but this does not mean that oPL is not present at earlier periods of pregnancy, because our RIA may not sensitive enough to detect smaller quantities of oPL. It is interesting that the secretion of oPL during pregnancy is similar to the pattern observed for human placental lactogen

and monkey placental lactogen, except that primate PLs do not decline until parturition(Belanger et al,1971; Spellacy et al,1972).

The oPL concentrations during pregnancy, measured by radioimmunoassay, cannot be compared quantitively with the oPL concentrations previously reported by radioreceptor assay (Kelly et al,1974; Djiane et al,1975; Handwerger et al,1975) since purified oPL and ^{125}I-oPL were not used in the radioreceptor assays. Handwerger et al (1975) reported that the oPL concentrations during pregnancy measured by radioreceptorassay were about twice those measured by radioimmunoassay. However, in their study, purified hPL rather than oPL was used as standard and ^{125}I-hPRL was used as tracer in the radioreceptor assay. Using ^{125}I-oPRL as tracer and oPRL as standard in the mammary gland receptor assay, Kelly et al (1974) noted peak oPL concentrations of about 1000-2000 ng/ml in three ewes during pregnancy and Djiane et al (1975) noted peak oPL concentrations of about 300 and 900 ng/ml in two pregnant ewes. Kelly et al(1974) and Djiane et al(1975) also noted wide variation in maternal oPL concentrations in the latter half of pregnancy and a decrease in oPL concentrations just before parturition.

It is interesting that the concentration of oPL in fetal sera is nine times higher than that in maternal peripheral sera from day 47-70. Although human PL and monkey PL are also found in the fetal circulation (Kaplan et al,1965; Friesen 1971), there are no reports that fetal PL levels are ever higher than maternal levels. Furthermore, there is almost no information available

on the role of PL in the fetus. Studies in our laboratory have shown that oPL binds to ovine fetal liver membranes and oGH competes for the same receptor sites as shown in Figure 21. This suggests that oPL may act as a GH in early gestation in the fetus. At later stages fetal pituitary GH may resume that role.

The presence of oPL in allantoic fluids is unexpected. The peak levels of oPL in allantoic and amniotic fluid are coincident with the transient peaks of estrogen-sulfate in amniotic fluid(private communications with Dr. H.A. Robertson). Whether this relationship is merely fortuitous or is related to steroidogenesis and placental attachment requires further examination.

2. Ovine Placental Lactogen in the Maternal and Fetal Cotyledons

It has been shown that the sheep corpus luteum (CL) is necessary for the maintenance of pregnancy during the first 50 days of gestation(Denamur et al,1955). The embryo also has been shown to be essential for the maintenance of the CL of pregnancy after day 12 (Moore et al,1966). The embryo may secrete a luteotropic substance which extends the life span of the CL rather than stimulating its secretory activity, especially as there is no evidence that the secretory activity of the CL increases during the first 50 days of pregnancy (Bassett et al,1969; Flylling, 1970). Whether the signal arising from the embryo is luteotropic or antiluteolytic is unknown. By RIA, oPL is detectable in the fetal chorionic membranes as early as 16-18 days of gestation. Thus,

it may be speculated that oPL may be a luteotropic hormone during pregnancy because it binds specifically to ovine CL membrane fractions as shown in Figure 20, but whether oPL actually stimulates progesterone secretion in the sheep is unknown.

Whether the high concentrations of oPL in the placentomes and in the circulation between 90 and 130 days of gestation are related to mammogenesis and lactogenesis is not clear, since we were not able to demonstrate any significant specific binding of oPL and oPRL to ovine mammary glands. However, it is known that the mammogenesis in ewes rendered hypoprolactinaemic is apparently normal (Djian et al,1975; Martal et al,1977) and that lactogenesis in the sheep mammary gland begins after Day 95 of pregnancy(Denamur,1965), the time at which there is a large increase in oPL production . Thus, it may be speculated that the mammogenesis and lactogenesis in the ewes are stimulated by oPL. oPL has been shown to have lactogenic activity in vitro by its effects on the histology of mammary gland tissue of pseudopregnant rabbits(Martal et al,1976) and on lactose synthetase activity and casein synthesis (Martal et al,1976; Chan et al,1976).

3. Half-time Disappearance Rate of oPL in the Sheep

Surgical method-

Our studies on the endogenous half-time disappearance of oPL calculated after the removal of the placenta is different from that of Handwerger et al (1977). Handwerger et al reported a monophasic disappearance of oPL with a half-time of 29.1 ± 1.3 min.

In our studies, we found that the endogenous half-time is approximately 50-57 min. The reasons for the discrepancy between Handwerger's results and ours is not clear at the present.

Injection method-

The multi-exponential curve observed for the disappearance of TCA-precipitable material over 240 min is similar to that for growth hormone (Frohman et al,1970), follicle-stimulating hormone(FSH)(Coble et al,1969; Butt et al,1973), Luteinizing hormone (LH)(Kohler et al,1968), hPL(Kaplan et al,1968; Reddy et al,1975). Higher levels of radioactivity found in the blood as compared with TCA-precipitable material suggests the presence of free ^{125}I in our ^{125}I-oPL preparation and also the presence of degradation products in later time.

The short half-time ($t_{\frac{1}{2}}$(s) on mean 1.5 min (range 1.3-1.7 min) for oPL decay as determined over the first 10-25 min is shorter than for growth hormone (5.7 min) (Frohman et al,1970) over 20 min. Neurophysin also has a relatively short half-time of

3.4 min(Forsling et al,1973) calculated by a method similar to that used in the present study.

The long half-time $t_{\frac{1}{2}}(L)$ of oPL calculated in this method is similar to the endogenous half-time disappearance rate of oPL calculated after removal of the placenta, approximately 50-65 min. Thus, our studies on the half-time disappearance rate of oPL using two different methods are in good agreement.

$t_{\frac{1}{2}}(S)$ is calculated by the formula of : $t_{\frac{1}{2}}(S)=0.693/\alpha$, where(α) is the slope of the regression line. Similarly, $t_{\frac{1}{2}}(L)$ is calculated from the formula of : $t_{\frac{1}{2}}(L)=0.693/\beta$, where β is the slope of the elimination phase curve. These formulae are adopted from Greenblatt et al(1975). Furthermore, we can calculate the "elimination rate constant"($K\varepsilon$) = $A + B / \frac{A}{\alpha} + \frac{B}{\beta}$ and the "clearance" formula of: Clearance = V_1 X $K\varepsilon$, where V_1 is the central compartment consists of serum or blood volume together with the extracellular fluid of highly perfused tissues such as the heart, lung,liver, kidney, and endocrine glands. Secretion rate = Plasma Concentration X Clearance.

4. Uptake of ^{125}I-oPL by Ovine Tissues in vivo

In the studies on the degradation of hPL in normal and nephrectomized dogs(Rochman et al,1972), and in normal and lactating rats (Reddy et al,1975), it was concluded that the kidney was the main site of uptake of the hormone. We also found that the kidney accumulated a high amount of radioactivity. This suggests that the kidney is involved in the uptake and presumably subsequent degradation of oPL. However, at present we don't known whether the hormone present in the kidney is still intact or not.

5.Relationships among oPRL, oGH, oPL and Progesterone in the Sheep during Pregnancy

In 1953 Sgouris and Meites postulated that prolactin may act to inhibit its own secretion by the pituitary . This hypothesis has been verified subsequently by many studies (for reviews, see Meites et al,1972; Clemens et al,1974; Tindal,1974). Recently, Nagasawa et al(1976) demonstrated that daily injections of human placental lactogen(hPL) significantly decreased both pituitary and serum levels of prolactin in the intact female rat . Their results suggest that hPL inhibits pituitary prolactin by increasing hypothalamic prolactin-inhibitory factor(PIF) activity. It is unlikely that hPL inhibits prolactin secretion by acting directly on the pituitary, since prolactin released by the pituitary into the medium was not affected by the addition of hPL. Nagasawa et al suggest that the inhibitory mechanism of hPL on pituitary prolactin secretion is similar to the negative short-loop of prolactin. The physiological significance of their finding on the interrelation between placental lactogen and pituitary prolactin is not clear at present.

Interestingly, the secretion of oPRL during pregnancy in sheep is similar to rats (Amenomori et al,1970; Nagasawa et al,1972). Furthermore, the levels of oPRL are inversely related to serum oPL and progesterone levels.

These studies raise questions as to whether the suppression of prolactin secretion during pregnancy is due to the high level of oPL in the circulation, and that the inhibitory effect of oPL on pituitary prolactin secretion is mediated by an increase in hypothalamic PIF activity. Whether progesterone also has a role in suppressing prolactin secretion during pregnancy in the sheep remains unclear.

E. BIOSYNTHESIS OF OVINE PLACENTAL LACTOGEN BY OVINE CHORIONIC MEMBRANES in vitro

It is apparent that, after gel filtration of the incubation medium, two radioactive peaks of proteins of widely differing molecular weight were precipitated by antisera to oPL. Since the molecular weight of highly purified oPL is 19,500 to 21,500 as shown in Figure 14, the total counts precipitated by anti-oPL in the original incubation medium as shown in Table VIII do reflect ^{3}H-oPL alone. The evidence presented suggests strongly that the molecular weight of the radioactive proteins which emerged in the second peak is identical with that of oPL. Accordingly, it is likely that the ^{3}H-oPL is contributing significantly to the second radioactive peak.

The nature of thelarge molecular weight species precipiated by anti-oPL is not yet clear. An alternative explanation is that these proteins might be aggregated oPL, or prohormone of oPL still attached to ribosomes, or there might be an oPL binding substance of large molecular weight present in placental tissue. Our studies clearly indicate and support the fact that oPL is synthesized and is secreted by ovine chorionic membranes.

F. BIOLOGICAL EFFECTS OF OVINE PLACENTAL LACTOGEN

Bioassay of Growth Promoting Activity of oPL

Bioassay of growth promoting activity using epiphyseal width increase in hypophysectomized rats is indeed the most specific assay. Although oPL promotes growth of hypophysectomized rats, its role in promoting growth of fetal lambs remains undefined. We have successfully demonstrated that oPL binds specifically to fetal liver and that high levels of oPL are in the fetal circulation. Moreover Handwerger et al (1976) have demonstrated that oPL increases somatomedin activity in hypophysectomized rat .

Luteotrophic Activity of Ovine Placental Lactogen

The corpus luteum of the rat is a complex gland which depends upon trophic hormones for continued existence and is under constant threat of destruction by lytic factors, such as prostaglandin. It is now generally accepted that the corpus luteum of the rat is under the control of both luteinizing hormone(LH) and prolactin and in the absence of either hormone its function is lost. Surprisingly, the requirement for each hormone by this gland changes with age and particularly with pregnancy (Morishige et al,1974) which may indicate a shift in functional control as a consequence of changes in the level of other lytic factors, such as prostaglandins(PG's).

Hichens et al (1974) showed that PGF_{2a} treatment of superovulated rats caused a marked drop in ovarian gonadotropin binding capacity, and this response was associated with a loss of LH-stimulable progesterone production and attenuation of cyclic AMP production in vitro (Grinwich et al,1975). Simultaneous treatment of the animals with prolactin blocked the effect of PGF_{2a} on serum progesterone and the LH-receptor when animals were sacrificed 24 h after PGF_{2a} treatment(Grinwich et al,1976).

In the present study simultaneous administration of lactogenic hormone (oPRL, oPL or hPL) prevented the loss of LH receptor and serum progesterone production by the PGF_{2a}. These

data argue for a close interaction between loss of corpus luteum LH-receptor and progesterone, and that the effect of lactogenic hormone is to prevent the loss of LH-receptor in the corpus luteum.

Treatment of rats with Bromoergocryptine produced a marked decrease in progesterone production and the LH-receptor. Bromoergocryptine suppresses prolactin secretion and simultaneous lactogenic treatment (oPRL, or oPL, or hPL) reversed the effect of ergocryptine on both the LH receptor and serum progesterone. It was of interest to observe that lactogenic hormone treatment elevated the number of LH-receptors by 1.5 fold. A previous report of Lee and Ryan (1975) showed that chronic treatment ofsuperovulated rats for 10 days with estrogen increased the capacity of ovarian tissue to bind gonadotropin. In the above report it was suggested that the effect may have been due to elevated prolactin. Certainly, the effect of estrogen on elevating circulating levels of prolactin in rats is well documented (Neill,1974). These reports in conjuction with the data in the present experiment, support the hypothesis that prolactin and placental lactogen may increase the number of LH receptors in the rat corpus luteum and prevent the decline in the number of LH receptors following treatment with PGF_{2a} or ergocryptine. It is possible that the mechanism of lactogen-LH interaction resides in the ability of lactogens to maintain the function of luteal cell LH-receptors.

G: COMPARATIVE DATA ON PLACENTAL LACTOGEN OF SEVERAL SPECIES

In the following discussion, I would like to summarize the data on the placental lactogen of several species into four tables and one figure. They are:

1. TABLE OF CHEMICAL PROPERTIES

2. TABLE OF IMMUNOLOGICAL PROPERTIES

3. TABLE OF BIOLOGICAL EFFECTS

4. TABLE OF SECRETION

5. SECRETION PATTERN OF PLACENTAL LACTOGEN DURING PREGNANCY

1. TABLE OF CHEMICAL PROPERTIES

SPECIES	HUMAN (hPL)	MONKEY (mPL)	SHEEP (oPL)	COW (bPL)	GOAT (cPL)	RAT (rPL)	MOUSE (moPL)	GUINEA PIG(gpPL)	HAMSTER (hamPL)
PURIFIED	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
AMINO ACID COMPOSITION	190[1]	183-185[2]	193[3]	-	-	-	-	-	-
MOLECULAR WEIGHT	21K[1]	21-22K[2]	21K[4]	22K[5];45K[6]	22K[7]	18-22K[8]	22K[9]	50-60K[9]	100K &[9] 18-22K
RELATIVE MOBILITY (R_fpH8.8-9.0)	0.72[1]	0.52[2]	0.39[3] 0.18[4]	0.52& 0.54[5]	-	-	-	-	-
ISOELECTRIC POINT	-	-	6.8[3] 8.8[4]	5.9[5]	6.4	6.5-7.0	-	-	-
AMINO ACID SEQUENCE	Known	No	No	No	No	No	No	No	No

[1]Sherwood et al (1971); [2]Shome et al(1971) [3]Handwerger et al(1974)
[4]Our studies(1976 & present) [5]Bolander et al(1976) [6]Roy et al(1977)
[7]Grissom et al(1977) [8]Robertson et al(1975) [9]Kelly et al(1976)

The molecular weight of hPL,mPL, oPL, bPL and rPL were estimated by SDS-polyacrylamide gel,whereas the molecular weight of cPL, moPL, gpPL and hamPL were estimated by gel filtration on Sephadex G-100.

2. TABLE OF IMMUNOLOGICAL PROPERTIES

ANTISERA	CROSS-REACTION WITH PLACENTAL LACTOGEN OF								
	HUMAN (hPL)	MONKEY (mPL)	SHEEP (oPL)	COW (bPL)	GOAT (cPL)	RAT (rPL)	MOUSE (moPL)	GUINEA PIG (gpPL)	HAMSTER (hamPL)
Anti-hPL	+[1]	+[1]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]
Anti-mPL	+[3]	+[3]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]
Anti-oPL	-[2]	-[2]	+[2,4]	-[2] +[5]	-[2]	-[2]	-[2]	-[2]	-[2]
Anti-bPL	N.R.*	N.R.	+[5]	+[5]	N.R.	N.R.	N.R.	N.R.	N.R.

*N.R.- No Report

[1]Josimovich et al(1964); Kaplan et al(1964); Friesen(1965); Grant et al(1970)

[2]Our own studies

[3]Shome et al(1971); Vinik et al(1973)

[4]Handwerger et al(1974)

[5]Bolander et al (1976)

Since purified cPL, rPL, moPL, gpPL, and hamPL are not available, we tested the placental extracts of these species only.

3. TABLE OF BIOLOGICAL EFFECTS

	HUMAN (hPL)	MONKEY (mPL)	SHEEP (oPL)	COW (bPL)	GOAT (cPL)	RAT (rPL)	MOUSE (moPL)	GUINEA PIG (gpPL)	HAMSTER (hamPL)
1.Lactogenic Effect									
(1)Pigeon Crop-Sac Assay	10%[1]		10%[2]						
(2)Rabbit Mammary Intraductal Assay	50-100%[3]		+[4]						
(3)Casein Synthesis	50-100%[3]		90-100%[5]						
(4)NAL-Synthetase Assay	14%[6]		25%[6]	4.5%[6]					
2.Somatotropic Effect									
(1)Body Weight Gain	<3%[7]								
(2)Tibial Width Assay	13%[1]	+[8]	>1.3U[5]	5%[6]					
(3)Uptake of Radioactive Sulfate by Rib Cartilages	+[9]		>1.3U[5]						
(4)Production of Somatomedin			+[10]						
3.Luteotropic Effect									
(1)Vaginal Mucinification Luteotropic Assay	+[11]	+[12]							
(2)Maintenance of LH-Receptor in Corpus Luteum	+[5]		+[5]						
4.Diabetogenic Effect (Lipolytic Effect)	+[13]								

[1]Li(1972) [2]Private communication with Dr. C. Nicoll, University of California, Berkely,U.S.A.
[3]Turkington(1971);Kleinberg et al(1971) [4]Handwerger et al(1974) [5]Our own studies
[6]Bolander et al(1976) [7]Josimovich et al(1962); Friesen(1965) [8]Friesen et al(1971)
[9]Kaplan et al(1964);Breuer(1969); Murakawa(1968) [10]Hurley et al(1977)
[11]Josimovich et al(1964); Kovacic(1966);Henzl et al(1970)
[12]Josimovich(1970) [13]Friesen(1965); Turtle et al(1966); Riggi(1966);Genazzani et al(1969)

4. TABLE OF SECRETION

	HUMAN (hPL)	MONKEY (mPL)	SHEEP (oPL)	COW (bPL)	GOAT (cPL)	RAT (rPL)	MOUSE (moPL)	GUINEA PIG (gpPL)	HAMSTER (hamPL)
1.Earliest Period detected oPL(day)									
(a)Maternal Peripheral Blood	30	40	48[1]	42[2]	60[4]	9[3]	8[3]	32[3]	7[3]
(b)Placental Tissue	18		16[1]						
2.Maximum Mat. Conc. (ug/ml)	3-10	3-7	0.6[1]	0.7-1.1[2]	1.6[4]	1.2[3]	1.2[3]	1.5[3]	2.5[3]
3.Foetal Conc. at Term(ng/ml)	<100	<100	50[1]						
4.Half-Time: $t_{\frac{1}{2}}(1)$	12	20	50-65[1]			12.5&[5]			
(minutes) $t_{\frac{1}{2}}(2)$	75	36 h				9.5			
5.Production Rate per Day (gram)	1	0.3	0.09[1]						
6.Placental Content (mg/g wet weight)	0.3	0.07	0.08[1]	0.05[3]	0.055[3]	0.045[3]	0.09[3]	1.0[3]	0.022[3]
Average Placental Weight (grams)	500	150	400[1]						
7.Placental Tissue Pool Turnover/Day	6	30	3[1]						

Table re-drawn from Friesen et al(1971) except that:

[1]Our own studies

[2]Bolander et al(1976)

[3]Kelly et al(1976)

[4]Currie et al(1977)

[5]Kelly et al(1974)

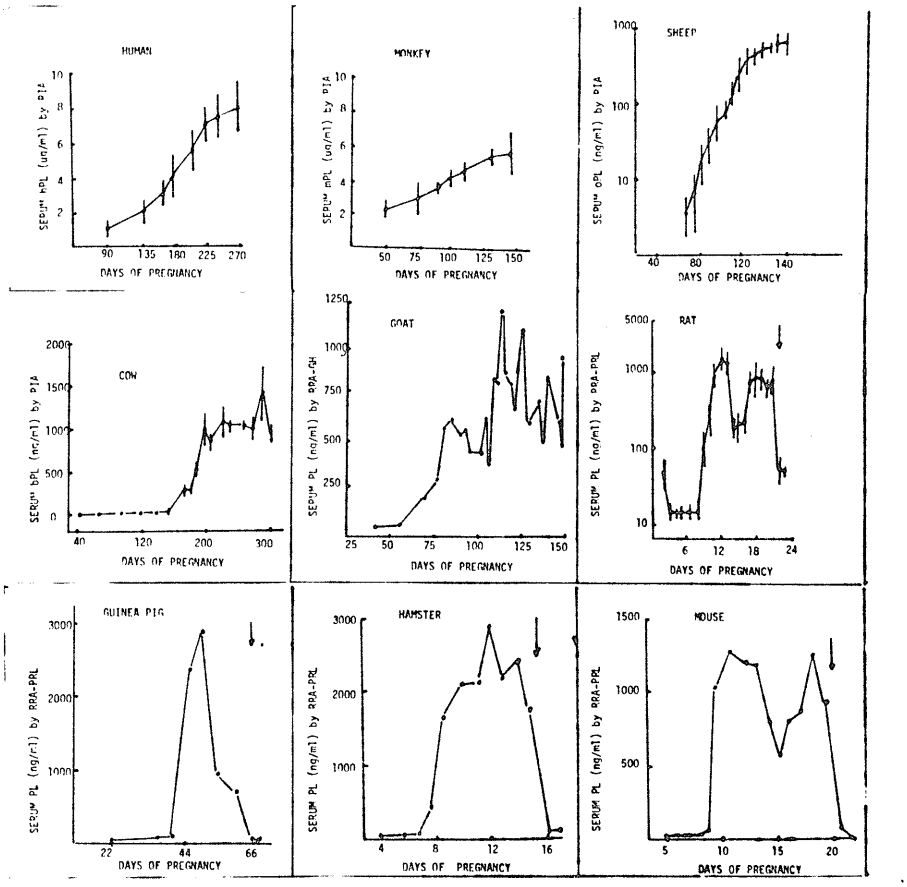

Figure 43. The secretion patterns of placental lactogen during pregnancy of human, monkey, sheep, cow, goat, rat, guinea pig, hamster, and mouse.
Human placental lactogen- redrawn from Friesen etal, 1971
Monkey placental lactogen- redrawn from Friesen et al, 1971
Ovine placental lactogen- redrawn from Chan et al, 1978
Bovine placental lactogen- redrawn from Bolander et al, 1976
Caprine placental lactogen- redrawn from Currie et al, 1977
Rat placental lactogen- redrawn from Shiu et al, 1974
Guinea pig placental lactogen- from Kelly et al, 1976
Hamster placental lactogen- redrawn from Kelly et al, 1976
Mouse placental lactogen- redrawn from Kelly et al, 1976

In conclusion, it is clear from the above data that a limited amount of information is available on non-primate placental lactogens for comparative studies. Until now, only hPL,mPL, oPL, bPL, cPL, and rPL have been purified or partially purified. Non-primate placental lactogens are immunologically different from primate placental lactogens. They all have a similar molecular weight of 20,000 to 22,000 with the exception of guinea pig and hamster and including of cow. Biologically, it appears that primate placental lactogens are less potent than purified oPL and bPL in both lactogenic and somatotrophic assays particularly when these hormones were tested simultaneously in the radioreceptor-assays for prolactin and for growth hormone. Furthermore, it is apparent that the secretion pattern of all placental lactogens are very similar in most species with the exception of mouse and rat and possibly the cow. In mouse and rat, two peaks of lactogenic activity were observed during pregnancy instead of one peak as seen in other species. Until now, no information is available on the amino acid sequence of placental lactogens with the exception of human placental lactogen which is 80% homologous with human growth hormone.

H. POSSIBLE ROLE OF OVINE PLACENTAL LACTOGEN IN THE SHEEP

The role played by oPL in the sheep during pregnancy is not understood. However, experimental evidence from several studies suggest that oPL may have three major roles in the sheep during pregnancy.

1. Mammotropic Effect:

The earliest experimental observation indicating that a placental hormone, possibly oPL, and progesterone, were involved in mammary gland development was reported by Denamur et al(1961). They found that hypophysectomy of pregnant ewes after 50 days of gestation did not affect the normal course of pregnancy. Although the mammary gland was not so well developed as in intact control, nevertheless some development did occur and transient lactation took place. Later, Djiane and associates found that when pregnant ewes were rendered hypoprolactinaemic by administration of ergocryptine, mammogenesis was still normal (Djiane et al,1975; Martal et al,1977). These studies suggest that the placenta might secrete a prolactin-like substance which is responsible for the normal mammogenesis in the ewes.

Ovine PL has been shown to have potent lactogenic activity by its effects on the histology of mammary gland tissue of pseudopregnant rabbits, on lactose synthetase activity and on casein synthesis (Martal et al,1976; Chan et al,1976). Furthermore, the

lowest levels of ovine pituitary prolactin and peak levels of oPL are found in the circulation at about 95 days of pregnancy, the time at which lactogenesis in the ovine mammary gland begins (Denamur,1965). Thus, from these observations, it may be speculated that mammogenesis and lactogenesis in the ewes are promoted by oPL. However, binding studies showed that less than 3% of specific binding of oPL was found in ovine mammary glands. Thus , it is possible that oPL may not have a direct effect on ovine mammary gland but has an indirect effect through other endocrine organs such as the ovary.

2. Metabolic Effect:

The only study to date which directly considers the metabolic effects of oPL in the sheep is that of Handwerger et al (1976). In their studies, they found that oPL administration caused an acute decrease in free fatty acid, glucose, and amino nitrogen concentrations. The effects observed were different from the effects observed for hPL in human. In man, intramuscular administration of hPL caused an increase in fatty acid concentration (Grumbach et al,1966). Thus, studies by Handwerger et al suggest that oPL is important in the modulation of intermediary metabolism during pregnancy but its effect may be opposite to the effect of ovine growth hormone in sheep.

Our studies on the binding of oPL showed that oPL binds effectively to ovine adipose tissues, and ovine growth hormone competes with oPL in a parallel manner in displacing the binding of ^{125}I-oPL to its receptor site. Thus, these studies suggest strongly that structurally the molecule of oPL is similar to oGH because they both compete with each other for binding to the growth hormone receptors in ovine tissues. Furthermore, sheep pituitary growth hormone during pregnancy is not elevated, whereas the substrate (glucose is the main source of substrates for the fetus) requirements by the conceptus progressively increases throughout gestation making it plausible that oPL acts as the "growth hormone" of pregnancy. If oPL acts as the "growth hormone" of pregnancy in the sheep, it might induce a tonic effect on maternal metabolism by mobilizing FFA release from adipose tissues for gluconeogenesis in the maternal liver which ensures the fetus a steady source of various fuels, of which glucose is the principal one. Insulin is a fluctuating modifier of the effect of oPL on the maternal organism. Feasting increases effective insulin and restores maternal substrates , whereas fasting results in a decreased effect of insulin and induces primary catabolic effects and gluconeogenesis enhanced by oPL to ensure an adequate supply of metabolic nutrients for the fetus.

3. Luteotropic Effect:

There are several pieces of evidence to support the view that oPL is the luteotropin in the sheep. Studies on hypophysectomy of pregnant ewes during pregnancy demonstrated that when hypophysectomy was performed in pregnant ewes before 30 days of gestation, the corpus luteum regressed within 10 days, and the embryo was eventually resorbed (Denamur et al, 1955;1966;1972; Amoroso,1952; Bjorkman,1965; Davies et al,1965; Boshier,1969). On the other hand, when the embryo is removed before day 12 of gestation, corpus luteum regressed within 2-3 days (Moore et al,1966). However, when hypophysectomy was performed on day 60 of gestation, the weight of the corpus luteum examined 12 days later, the weight of corpus luteum remained at 75% of the control value, and abortion did not occur. Our studies indicate oPL is detectable in uterine vein blood after day 25 of gestation, the time when the conceptus acquires its luteotropic properties. Thus these studies strongly indicate that the corpus luteum is necessary for the maintenance of pregnancy during the first fifty days of gestation, the embryo is essential for the survival of the corpus luteum of pregnancy after day 12, and oPL may be the luteotropic hormone during pregnancy.

Another piece of evidence suggesting that oPL is the luteotropic hormone in sheep is derived from studies on the binding of oPL to ovine corpus luteum. We have demonstrated that oPL binds specifically to sheep corpus luteum. Thus, it is conceivable

through its binding to corpus luteum, that oPL may exert an action on the corpus luteum. In fact, our studies on the biological effect of oPL using pseudopregnant rat corpora lutea demonstrated that oPL has the ability to prevent the loss of LH-receptor in the corpora lutea and the fall of progesterone in the circulation. In short, our studies in conjunction with other studies strongly suggest that oPL may be a luteotropic hormone during pregnancy. The role played by oPL in the sheep during pregnancy is to prevent the loss of or possibly to increase LH-receptors in the corpus luteum. Furthermore, oPL may also have the role in blocking the luteolytic effect of prostaglandin (PGF_{2a}) during early pregnancy.

In conclusion, the physiological roles of oPL in the sheep remain unclear, but three possible effects may be mentioned: namely, the stimulation of mammary gland growth, adaptation of metabolic processes during pregnancy, and finally luteotropic effects.

I. POSSIBLE EXPERIMENTS TO DEMONSTRATE THE BIOLOGICAL EFFECTS OF OVINE PLACENTAL LACTOGEN IN SHEEP

1. Mammotropic Effect:

The mammotropic effect of oPL in sheep may be demonstrated by the effects of oPL on lactogenesis in explant cultures of mammary gland tissues from early pregnant ewes according to the method described by Jeulin-Bailly et al(1973) and Collier et al(1977). If the availability of ovine placental lactogen permits, one may examine the effects of oPL on the morphology of the ovine mammary glands after chronic administration of oPL into the ewes which have primed with steroids. The methodology will be similar to that described by Beck(1972).

2. Metabolic Effect:

The metabolic effects of oPL may be demonstrated using in vitro and in vivo systems. The effect of oPL on lipolysis and incorporation of amino acid could be studied using the method described by Friesen(1965), Turtle et al(1966), Riggi(1966), Genazzani et al (1969), except that ovine tissues are used instead of those from rat or rabbit.

If the availability of oPL permits, oPL may be infused into early pregnant ewes for a short period in order to observe the effects of oPL on the levels of several metabolites, such as FFA, glucose, and amino nitrogen in the circulation.

Furthermore, oPL measurement in sheep serum during various physiological conditions, such as restricted feeding during pregnancy or starvation, may provide insight into the possible role of oPL.

3. Luteotropic Effect:

The luteotropic activity of oPL in the sheep may be demonstrated by several means. First, active immunization of the non-pregnant ewes with oPL before mating could provide evidence to indicate whether oPL is essential for normal pregnancy. Secondly, by measuring plasma levels of progesterone and by examining the pregnancy after infusion of oPL-antibodies into early pregnant ewes one might obtain evidence to indicate that oPL is essential for the maintenance of early gestation. Finally, if the availability of oPL permits, oPL may be infused into the cycling ewes on day 12-13 of the estrus cycle to examine whether the life-span of the corpus luteum could be extended or saved.

SECTION VII SUMMARY

In summary, we have been successful in purifying ovine placental lactogen from ovine placental cotyledons. Binding studies of oPL on ovine tissues showed that the distribution of binding sites for oPL are located on liver(maternal and fetal), adipose tissue, ovary, corpus luteum, and non-pregnant uterus. Kinetic analysis showed that the binding of oPL to the receptor occurs readily at physiological concentrations of the hormone. Specificity studies showed that only growth hormone proparations could displace oPL binding to its receptor sites whereas other hormone preparations could not, indicating that oPL binds specifically to GH-receptor sites in the sheep tissues.By employing the radio-receptorassay for placental lactogen using oPL and sheep liver as hormone standard and receptors respectively, placental lactogens were detected in the placental extracts of human, monkey, goat, guinea pig, and mouse. Placental lactogen could not be detected in placental extracts of pig, dog, horse, cow, rat, and rabbit.

A specific homologous radioimmunoassay for oPL(RIA-oPL) has been developed. The assay is specific for oPL in that oPRL,oGH, and as well as other hormones including rat, caprine, bovine, monkey and hPL exhibited no cross-reaction in the assay. Utilizing this RIA, oPL is detected in uterine vein blood samples as early as 25 days of gestation. The secretory pattern of oPL is similar to that of human and monkey placental lactogen. oPL is found in the

circulationand allantoic fluids throughout gestation with high levels during early pregnancy. oPL is not detectable in amniotic fluid and maternal urine. oPL is detectable in extracts of chorio-allantoic membranes and maternal caruncles as early as 16 days of gestation with higher concentrations in fetal membranes.The concentration and content of oPL in placentomes reaches peak levels around day 80. When oPRL and oGH were measured by specific RIA in the maternal circulation during pregnancy, the secretory pattern of oPRL is inversely related to that oPL secretion whereas there is no significant variation of oGH levels. This finding suggests that oPL might be the hormone that suppresses oPRL secretion during pregnancy. The half-time disappearance rate ($t_{\frac{1}{2}}$) of oPL in the sheep is approximately 60 minutes. This indicates that the daily production of oPL during late gestational period is approximately 90-100 mg. Furthermore, biosynthesis studies showed that oPL is synthesized and secreted by the chorioallantoic membrane as early as day 22. This study demonstrates that oPL is of embryonic origin.

In bioassays, we are able to demonstrate that oPL is able to stimulate growth of epiphyseal cartilage of hypophysectomized rats. We are able to demonstrate that administration of oPL into pseudopregnant rats prevents the loss of LH-receptors in the corpora lutea and fall of progesterone caused by PGF_{2a}. These studies suggest that the roles of placental lactogen during pregnancy is to act as the "growth hormone" of pregnancy and to maintain the integrity of LH-receptors in the C.L..

SECTION VIII SIGNIFICANCE OF PRESENT STUDIES

1. With the identification and isolation of oPL from sheep placentas we have demonstrated unequivocally that placental lactogen is present in non-primates.
2. We have established a method for the purification of oPL. By employing this method, one can purify oPL in large quantities and in high purity for chemical and biological studies.
3. We have developed a specific radioimmunoassay for oPL. By employing this assay, one can study the physiology of oPL in the sheep.
4. We have demonstrated for the first time that oPL can maintain the integrity of LH-receptors in the corpora lutea.
5. Binding studies of oPL using animal and human tissues show that oPL and hGH bind to the same receptor sites in both animal and human tissues, suggesting that the binding sites in oPL and hGH molecule are very similar. Thus, further structural analysis on the active sites of oPL and hGH will elucidate the peptide(s) which may be responsible for the binding for growth promoting activity. Hence, structural analysis may have potential implications for clinical use.

SECTION IX PUBLICATIONS

A) Manuscripts:

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