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PURIFICATION AND CHARACTERIZATION OF 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

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To

My parents and Fiancée:

Mr.& Mrs.Kai-On Chan

and

Miss.Ting-Mei Feng

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF OVINE PLACENTAL LACTOGEN. John S.D. Chan, Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada.

A placental hormone, ovine placental lactogen(oPL), has been identified and measured in serum and placental extracts at different stages of pregnancy using radioreceptorassays for prolactin (RRA-PRL) and for growth hormone (RRA-GH) to monitor the hormonal activities. Using conventional protein purification procedures, oPL was purified 1,000-fold from sheep cotyledons. The molecular weight of oPL is similar to human growth hormone (hGH) approximately 22,000 and its isoelectric point is 8.8 as determined by isoelectric focusing. A radioimmunoassay for oPL has been developed in which sheep pituitary growth hormone and prolactin as well as other pituitary and placental hormones from several species exhibit no cross-reaction. In the 2 radioreceptorassays (RRA's), the displacement curve of oPL is parallel to ovine prolactin (oPRL) and hGH standards and the ratio of prolactin activity to growth hormone activity (PRL/GH) of oPL is 2:1 as compared with a ratio of 1:1 for hGH and 100:1 for human placental lactogen (hPL). In a hypophysectomized rat weight-gain bioassay, oPL is 1.5 times more potent than a bovine growth hormone (0.9 U/mg) standard.

In rabbit mammary gland explants, oPL is equipotent with ovine prolactin standard (NIH-P-S-10, 26 U/mg) in stimulating casein synthesis. Non-primate growth hormone preparations cross-react only with labelled-GH in the RRA-GH, but not with labelled-PRL in the RRA-PRL, whereas hGH and oPL cross-react equally well in both RRA's. The similarity with which oPL and hGH cross-react in the 2 RRA's suggests that structurally oPL may resemble hGH more closely than hPL and non-primate pituitary growth hormone preparations. This hypothesis was supported by the finding that oPL is able to bind to growth hormone-receptors in human tissues, whereas non-primate growth hormones do not. Human tissue receptors for growth hormone fail to distinguish between oPL and hGH, suggesting that the active sites of oPL and hGH are similar, and raising the possibility that oPL may potentially have therapeutic use in the treatment of growth hormone-deficient patients.

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particularly to Mr. Jeff Harris and Mrs. Helle Cosby , for their co-operation and technical assistance.

CONTENT

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

Others:

^{o}C	Degree Centigrade
cpm	Counts Per Minute
g	Gram
mg	Milligram
ug	Microgram
M	Molar
mM	Millimolar
ml	Milliliter
ul	Microliter

N	Normal
ng	Nanogram
l	Liter
g	Gravitational force
IU	International unit
U	Unit

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SECTION I: GENERAL INTRODUCTION

The placenta occupies a unique position among the endocrine glands. It is a temporary organ with a genetic constitution which is partly foreign to the mother. It thus constitutes a natural homograft which, however, for a prolonged period of time escapes immunological destruction. During this time it plays an important role in the endocrine changes of pregnancy, producing hormones with a profound metabolic influence on the mother, yet it operates with a high degree of autonomy and apparently is independent of ordinary homeostatic and regulatory mechanisms in the maternal organism. One of the best known capabilities of this highly developed, though transitory, tissue is the maintenance of foetal-maternal gradients of nutrients, gases, and metabolites which are favorable to the foetus. Less well understood, however, is the role played by the placenta as an endocrine organ, and the physiological significance of placental proteins. 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. For example, the method of total extirpation and replacement treatment, is denied to investigators of placental endocrinology. Nevertheless, such problems are not a prohibitive barrier to investigation; and indeed, in recent years there has been an increasing interest in this facet of reproductive physiology.

Brief review on the early investigations of the endocrine placenta

Early events in the study of the placenta as an endocrine gland date back more than 70 years when Bouchacourt in 1902 (Bouchacourt,1902) treated women with oral doses of "chorinine", an extract of sow placenta, in order to stimulate lactation. In 1905 Halban (Halban,1905) on the basis of clinical observations that ovariectomy did not terminate pregnancy in human, suggested that the placenta may act as an endocrine organ. Moreover, Starling (1905) found that extracts prepared from the rabbit foetus produced mammary growth when injected into virgin females. Hence both concluded that mammary development in pregnancy was controlled by a substance secreted by the placenta. Later in 1913 Aschner (Aschner,1913) also reported that placental extracts have luteotropic effects when injected into lower animals.

Discovery of human chorionic gonadotropin (hCG)

In the two decades following the publication of Halban's view on 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. Subsequent studies by Evans and co-workers (Evans et al,1933 and 1935) demonstrated that urinary gonadotropins were found in patients with embryonic neoplasia. Their findings lent important 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 1930's, it was called "prolan". Because of its ready availability in copious quantities from urine of pregnant women, it has been studied in terms of its physiological, biological, and immunological properties. A great deal of information is available concerning hCG but its biological role in human pregnancy is yet to be defined clearly. Highly purified hCG is known to be a glycoprotein of 36,000 - 40,000 M.W. consisting of 2 subunits, alpha and beta. The latter is responsible for its hormone specific effects (Bahl,1972;Morgan, 1972). HCG is known to provide interstitial cell stimulating hormone (ISCH)-like activity and perhaps to have a relatively small amount of follicle stimulating (FSH)-like activity (Albert and Derner,1960; Channing,1970; Flint and Armstrong, 1972). This hormone is present in the maternal circulation long past the time when the ovary contributes significantly to circulating hormonal steroids. Therefore, it has been

suggested that its role as a gonadotropin is a transitory one. The continued production of hCG beyond the point at which there is need for conceptus to stimulate the ovary to provide for optimum steroid synthesis could simply represent a vestigial function. Still to be investigated thoroughly is the possibility of an autoregulatory, or at least an autostimulatory role, for the placenta through hCG on its important steroid synthetic function.

Discovery of human placental lactogen (hPL)

At the time that "prolan" or hCG came to the attention of reproductive physiologists in 1930's, Madruzza in 1927 (Madruzza,1927) reported that when placental homografts were implanted into virgin guinea pigs lactation ensued, suggesting that placentas contain substances other than gonadotropins. Subsequently, studies notably by Ehrhart (1936), Coppedge and Segaloff (1951), Gemzell et al (1955), Lyons and coworkers (1955), Ito and Higashi (1961), Fukushima (1961), Kurosaki (1961), all 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 sommatomammotropin (hCS), that there was renewed interest in the placenta as an endocrine organ. The discovery of hPL by Josimovich and MacLaren was based

on the finding that the human placental extracts partially cross-reacted with hGH antiserum in a double diffusion system. There is now a large literature on hPL. Thus in this section, attempts will be made to outline the evidence of placental lactogen production in mammals generally (selecting few animals as a representative discussion),and to assess the biological activity and possible functions of the hormone. The general term " placental lactogen " here is used to denote a protein hormone secreted by the placenta which is distinct from chorionic gonadotropin (hCG) and which exhibits some of the major effects described by Josimovich and MacLaren (1962).

Comparative assessment of placental lactogen production and effects in three orders of mammals

Order Primates:

Human placental lactogen (hPL)

Chemistry-

Human placental lactogen (hPL) or human chorionic somatomammotropin (hCS) is a single chain polypeptide with 190 amino acids, has a molecular weight of 21,500 with N-terminal valine and C-terminal phenylalanine held together by 2 disulfide bridges (Friesen,1965; Andrew,1969; Li et al,

1971; Sherwood et al,1971). Its complete amino acid sequence has been determined (Sherwood et al,1971; Niall et al,1971; Li et al,1971) and shows a remarkable similarity to that of human growth hormone (hGH). Over 80% of the residue positions are occupied by identical amino acids in the two hormones (Sherwood et al,1970; Li et al,1971; Bewley,1971; Niall et al,1971) and, if acceptable replacements are also considered, the extent of the homology becomes more than 95%. Marked similarities also become apparent between these hormones and the growth hormones and prolactins of ruminants (Wallis, 1971; Handwerger,1974; Bewley and Li,1974). Only recently human prolactin has been isolated and clearly differentiated from hGH (Lewis et al,1971; Hwang et al,1972) and preliminary structural studies (Niall et al,1972) indicate that it is more closely related to ovine prolactin than to hGH, but nevertheless it is very clear that all these hormones represent closely related group of molecules. The detection of internal homologies, that is repeating areas of similar sequence, within the molecules of hGH, hPL, ovine growth hormone and prolactin has led to the suggestion that these hormones evolved from a primordial peptide by gene duplication (Bewley and Li,1974).

Biological effects.

Human placental lactogen has minimal prolactin-like activity in the pigeon crop-sac assay, about 10% the potency

of sheep prolactin (Forsyth,1970; Li,1972), but a high level of activity in stimulating the mammary gland. Various assay systems have been used, including intraductal injection of hormone into the mammary glands of pseudopregnant rabbits (Forsyth,1970) and induction of casein synthesis(Turkington, 1971) or histologically assessed secretion (Forsyth,1971) in midpregnant mouse mammary gland in vitro. In such systems hPL shows from 50 - 100% of the activity of sheep prolactin (Forsyth,1971; Turkington,1971; Kleinberg et al,1971). HPL is also luteotropic in mice (Kovacic,1966) and in rats (Josimovich et al,1963), although no steroidogenic effect of hPL on the human corpus luteum could be demonstrated when it was given together with human chorionic gonadotropin (hCG) (Stock et al,1971).

Despite the remarkable similarity of hGH and hPL in primary structure, the two hormones have very different growth promoting effects or potencies, Li (1972) has reported that hPL has 13% of the activity of hGH in the tibial test, while the results of other authors indicate lower activity of less than 3% (Josimovich and MacLaren,1962; Friesen,1965), and it is generally agreed that growth promoting potency of hPL in man is minimal (McGarry and Beck,1972). Nevertheless, the hormone has important metabolic effects on protein, carbohydrate , and fat metabolism in various systems (Hartog, 1972).

The possible role of hPL in human is not clear,

however, Grumbach and coworkers (Grumbach et al,1968; Grumbach and Kaplan,1974) have developed the hypothesis that hPL which is secreted into the maternal circulation affects the metabolism of the mother in such a way as to ensure adequate supplies of glucose, amino acids, and minerals for the developing foetus. This concept has recently received support from experiments in which hPL was given to pregnant rats, or to foetuses (Mochizuki et al,1972). HPL given to the mother increased fetal body weight, total glycogen, and triglycerides, but has no effect when given directly to the foetus.

Thus, although the functions of hPL are not fully understood, the concept is emerging of a hormone which could be of 3-fold importance to the foetus, namely: 1) In assisting the maintenance of pregnancy by a luteotropic effect. 2) In promoting fetal growth by an effect on the metabolism of the mother. 3) In assisting the development of the mammary gland for lactation.

Immunological studies.

HPL cross-reacts immunologically with hGH and with monkey growth hormone (mGH) and monkey placental lactogen (mPL) (Josimovich,1964; Friesen,1965; Kaplan,1971; Grant et al,1970; Shome and Friesen,1971; Vinik et al,1973). An earlier report indicated some immunological cross-reaction between antiserum to hPL and placental extracts from a number

of species including monkey, rat, dog, pig, horse, sheep, rabbit, and cow (Gudson et al,1970), suggesting that there is placental lactogen in other non-primate species. However, this has not been confirmed.

An interesting novel effect of hPL and hCG on lymphocyte transformation in vitro has been reported recently (Contractor, 1973). HPL at concentrations similar to those found in late pregnancy caused a marked inhibition of the reaction. On the basis of this and other evidence the authors raised the possibility that hPL may play a role in blocking maternal immunological responses directed against the foetus. While this undoubtedly is an interesting hypothesis it seems unlikely that this mechanism is the sole reason for the "tolerance" of the foetus by the mother.

Secretion-

Using immunofluorescence-labelled antibody techniques, it has been demonstrated that hPL is secreted by the syncytiotrophoblast (Beck,1967; Sciarra et al,1963; Beck,1970), and has been detected in 12 to 18 day old embryos (Beck,1970).

Levels of hPL in the maternal circulation rise progressively until 34 to 36 weeks of pregnancy and then decline slightly (Spellacy,1972). At term, the concentration is as high as 5 - 10 ug/ml in the mother, however, levels in the foetus are less than 1% of maternal values, less than 100ng/ml (Kaplan and Grumbach,1965). There is virtually no information

on the factors which regulate the secretion and synthesis of human placental lactogen.

Monkey Placental lactogen (mPL)

The discovery of mPL was first reported by Kaplan and Grumbach in 1964 who demonstrated that monkey placenta contains a substance that cross-reacts with antisera to hGH and hPL. Based on this unique property of mPL, mPL has been purified and characterized by several investigators (Shome & Friesen,1971; Grant et al ,1970).

Chemistry-

Monkey placental lactogen (mPL) consists of two components, mPL-1 and mPL-2 of molecular weight 21,000 and 22,500 respectively. Amino acid compositions of mPL-1 and mPL-2 are very similar as compared with those of hPL and hGH.

Biological effects-

In limited studies on the somatotropic activity of mPL (Shome and Friesen,1971), mPL appeared to have a greater growth-promoting activity than hPL but less than pituitary growth hormone as shown in Figure I. MPL was also found to have a biologic potency of the same order of magnitude as hPL in the mouse vaginal mucification luteotropic assay

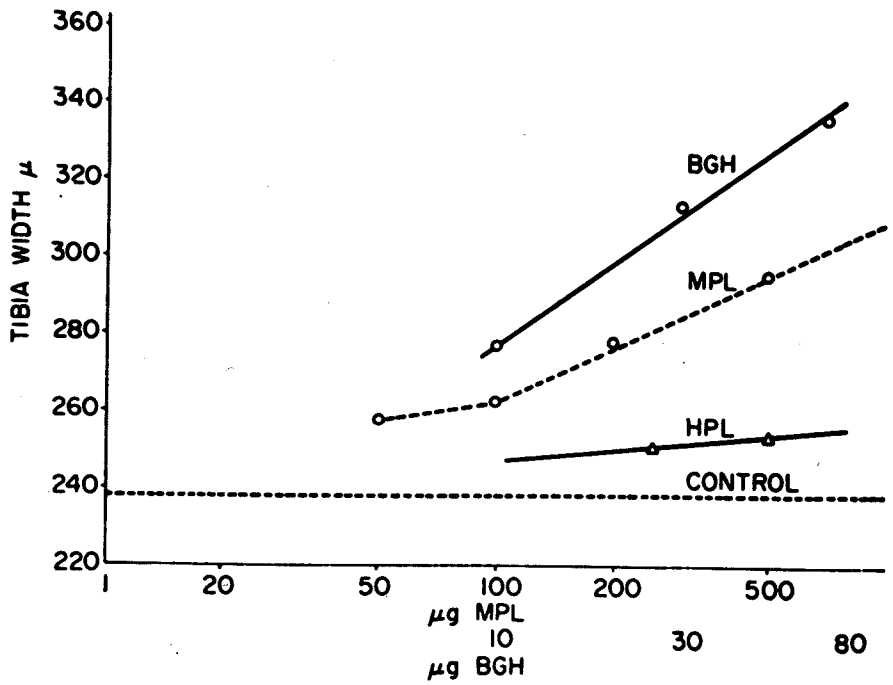

Figure 1. Epiphyseal cartilage width in hypophysectomized rats after administration of bGH, mPL or hPL for 4 days. The daily dose administered is indicated in the abscissa which is a 2 cycle log scale. The units for mPL and hPL are 10 fold greater than for bGH. Each dose was tested in 8 rats. The horizontal interrupted line is the cartilage width of control rats receiving only saline (from Friesen et al,1971).

(Josimovich, Wilson, and Leff,1970).

Immunological studies.

From the studies on the immunochemistry of mPL, hPL, and hGH, MPL cross-reacts to a greater extent with antisera to hGH than does hPL (Belanger et al,1971). Extracts from all monkey species studied cross-reacted equally using a radio-immunoassay for mPL. However, pituitary prolactins do not cross-react in the assay system (Friesen et al,1971).

Secretion.

The synthesis of mPL has been studied by Friesen (1968), who suggested that both forms of placental lactogen were synthesized in monkey placentas.

The secretion of mPL and hPL proved very similar; the concentrations of placental lactogen increased throughout pregnancy to reach a mean concentration of 6 ug/ml., whereas umbilical vein mPL was less than 100 ng/ml.. After delivery, maternal mPL concentration decreased rapidly with a half-time disappearance rate of 20 min.(Belanger et al,1971)as compared with 1/2 life of 13 min. for hPL. The second 1/2 life of mPL is 36 hours as compared with 40 min. 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 (ug/g dry weight) is 10% that of hPL (Kaplan and Grumbach,1971).

Experiments of foetoectomy performed by Friesen et al (1971) demonstrated that foetus did not exert any acute control over mPL secretion, 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.

Order Rodentia

Some of the earliest studies indicating production of a prolactin-like hormone by the placenta were done in the mouse (Newton and Beck,1939; Nandi,1959; Cerruti and Lyons, 1960; Choudary and Greenwald,1969; Komoto and Bern,1970); in rat (Pencharz and Lyons,1931; Selye, Collip, and Thompson, 1933; Astwood and Greep,1938; Lyons,1944; Averill, Ray, and Lyons,1950; Ray, Averill,Lyons, and Johnson,1955; Matthies, 1965,1966,1967,1971,and 1974), in guinea pig (Pencharz and Lyons,1934; Nelsons,1935; Amoroso and Finn,1962; Heap and Deanesly,1966; Heap, Perry, and Rowlands,1967), and in hamster (Talamates,1973) but in neither species has the hormone been isolated. Of these animals, rat placental lactogen (rPL) or rat chorionic mammotropin (rCM) is the one which has been most intensively characterized by classical bioassay. Therefore, in the following discussion, I wish to confine discussion to rat placental lactogen.

Rat Placental lactogen (rPL)

The ability of the rat to maintain the gravid state when hypophysectomized during the second half of pregnancy was first demonstrated by the work of Pencharz and Lyons (1931) and Selye, Collip, and Thompson (1933). These authors described the twelfth day of pregnancy (day 12) as a " critical period " for hypophysectomy in this species because pituitary ablation before day 12 resulted in the termination of pregnancy. Utilizing the deciduoma reaction, Astwood and Greep (1938) demonstrated that progesterone-secreting function of the copora lutea of the pseudopregnant rat could be maintained to the seventeenth day of pseudopregnancy by injection of extracts of rat placenta from eighth to sixteenth day of pregnancy. These authors also demonstrated that deciduoma could be produced in hypophysectomized pseudopregnant rats given extracts of rat placenta as source of this activity. Lyons (1944) showed that rat placental extracts are capable of synergizing with ovarian steroids to induce mammary lobulo-alveolar growth and early indications of lactation. Averill, Ray, and Lyons (1950) assayed rat placental tissue at various stages of pregnancy by implantation into pregnant rats hypophysectomized on day 6. The presence of viable embryos at autopsy on day 12 was considered evidence of the luteotropic activity in the placental tissue. The stage of gestation at which placental extracts provided the greatest

effect was day 12. Ray, Averill, Lyons, and Johnson(1955) demonstrated by means of assay of its various components, that the most potent luteotropic and mammotropic activity was found in the trophoblastic tissue from day 12 placenta. Desjardin and coworkers (1968) demonstrated that removal of the foetus plus foetal placentae on day 12 or 16 of pregnancy reduced the weight of mammary glands to control, non-pregnant levels by day 21. Removal of the foetus alone on day 16 had no significant effect on mammary weight, DNA, or RNA content on day 21. While removal of the foetus on day 12 did depress all these parameters to some extent, considerable mammary development had nevertheless occurred between days 12 and 21. Moreover, the DNA and RNA content of the mammary gland were 30 and 40% lower, respectively, in pseudopregnant as compared with pregnant rats on day 12. The placenta, therefore, appears to make some hormonal contribution to mammary development before mid-pregnancy and to provide the major stimulus in the second half of pregnancy. Similarly, Matthies (1965,1966,1967,1971) demonstrated the presence of luteotropic and mammotropic factors in foetal placental and maternal serum of day 12 pregnant rats hypophysectomized and ovariectomized on day 5. Ovarian interstitial tissue, adrenal cortex, and thyroid were reported to be unresponsive to the injection of placental extracts.

Placental involvement in extrauterine weight gain in pregnant rats is also suspected. The nature of the weight

gain is not fully understood, but water retention is involved (Brooksby and Newtons,1938; Dewar,1957). In mice, body weight is unaffected by hypophysectomy at mid-pregnancy (Newton and Beck,1939) and by the destruction of foetuses, but weight loss occurs when placentae are delivered (Brooksby and Newton, 1938). Progesterone maintains body weight in ovariectomized, hysterectomized, pregnant females and, in non-pregnant mice, mimics the weight increase of pregnancy. Thus, progesterone secretion, maintained by the luteotropic effects of placental lactogen, is probably largely responsible for the increased body weight of pregnant mice (Dewars, 1957).

Matthies in 1967 reported that rat placental extracts have minimal effect in pigeon crop-sac bioassay, and showed no growth-promoting activity using tibial epiphyseal cartilage-width bioassay. At the moment, it is difficult to implicate rat placental lactogen as the hormone that mediates the metabolic change occuring in the rat during pregnancy. During pregnancy, there is a decreased carbohydrate tolerance with lack of insulin sensitivity (Knopp et al,1970), despite increased circulating concentrations of insulin (Herrera et al,1969) which progresses as pregnancy advances. Thus, rat placental lactogen might mediate some of the metabolic changes of pregnancy.

The data provided by the investigators cited above indicates that the rat placental hormone possesses luteotropic and mammotropic acivities and is present in trophoblastic

elements of the midpregnant rat placenta and in maternal peripheral blood, The highest concentration of this hormone in maternal serum occurs on the 12th day, and was thought not to be present thereafter, as judged by the relatively crude bioassay method used. However, following the demonstration of rat placental lactogen using radioreceptorassay method (see section on NEW DATA ON NON-PRIMATE PLACENTAL LACTOGENS) Matthies now has confirmed using classical bioassays that rat placental lactogen is found in serum of day 19 pregnant rats.

Order Artiodactyla

This order is divided into three suborders, the Suiformes, the Tylopoda, and the Ruminantia. Hypophysectomy during pregnancy and co-culture experiments have demonstrated that a placental lactogen is secreted by four species in the last suborders, the goat (Cowie et al,1963; Cowie and Tindal, 1971; Buttle et al,1972; Forsyth,1972; Forsyth and Buttle, 1972; Forsyth,1974), cow (Forsyth and Buttle,1972), sheep (Denamur and Martinet,1961; Cowie et al,1963; Forsyth,1974), and the deer (Forsyth,1974). Of these animals, only goat placental lactogen is the one which has been most intensively studied and characterized by the classical endocrine methods, and therefore, in the following discussion I will limit it

to goat placental lactogen.

Goat placental lactogen (cPL)

The integrity of the pituitary is essential for the maintenance of pregnancy in some species but not in others. We can place the goat in the former category and the sheep in the latter. In 1963, Cowie and coworkers 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,1951). However, pregnancy after ovariectomy can be maintained in the goat by progesterone administration (Meites et al,1951). It is probable therefore that abortion occurs after hypophysectomy and pituitary stalk section because these operations result in an acute deficiency of luteotropic hormones, which in turn leads to dysfunction of the luteal tissue and a lack of progesterone (Cowie et al,1963).

The growth and differentiation of the mammary gland during pregnancy is a response to the action of mamy 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 a radioimmunoassay and for total lactogenic activity by a rabbit mammary gland organ

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. Thus, this discrepancy in the results of the two assays suggested that the blood of pregnant goats contains a second lactogen of placental origin which does not cross-react immunologically with pituitary prolactin.

Co-culture experiments (Forsyth,1972) demonstrated that this material is secreted by foetal cotyledons, and could be detected in the maternal circulation from the 9th week (64th day) 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 stage of pregnancy this hormone is first secreted.

As judged by gel filtration experiments, the molecular weight of goat placental lactogen is estimated to be 20,000 (Forsyth and Myers,1971), which is close to the molecular weight of hPL and mPL. It shows no significant cross-reaction with ovine prolactin (Buttle, Forsyth, and Knaggs, 1972). Apart from its ability to stimulate secretion by mouse and rabbit mammary gland in vitro, little else is yet known of the properties of goat placental lactogen.

The physiological roles of goat placental lactogen during pregnancy are unknown. However, Cowie (1970) has shown that there is a rapid growth of lobulo-alveolar tissue in the mammary glands of goats between the 70th and 100th

days of pregnancy, so the appearance of this second lactogen coincides with the period of intensive lobulo-alveolar development. The latter can be produced in non-pregnant goats by inducing prolactin release by repeated application of the milk stimulus (Cowie, Knaggs, Tindal & Turvey, 1968), but the maximum development obtained did not approach that occuring in pregnancy. Similarly, some mammogenesis can be obtained in ovariectomized and hypophysectomized goats by the injection of hexoesterol, progesterone, corticotropin, growth hormone and prolactin (Cowie, Tindal & Yokoyamo,1966), but again the lobulo-alveolar development did not approach that occuring in pregnancy. Furthermore, the concentration of prolactin (RIA) throughout gestation was low and thus prolactin would not appear to be the major mammotropic hormone responsible for the development of the mammae during pregnancy. Therefore, the high levels of placental lactogen may largely be responsible for lobulo-alveolar growth in goats.

SECTION II NEW DATA ON NON-PRIMATE PLACENTAL LACTOGENS

Brief introduction of a new assay system - radioreceptorassay (RRA).

Until recently, only human and monkey placental lactogen (hPL and mPL) were fairly well defined. Because of the ethical problems of performing experiments on placental lactogen in humans, the identification and characterization of placental lactogens in non-primates is a subject of very active investigation. A great impetus to this research both in our laboratory as well as others has been the development of two radioreceptorassays in the past two years. The one measuring prolactin or lactogen (RRA-PRL) utilizes a rabbit mammary gland receptor (Shiu et al,1973) while the one for growth hormone-like activity employs a rabbit liver receptor (Tsushima & Friesen,1973).

The development of receptor assays 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, 50 ng/ml for serum samples), and most importantly, they are

not species specific. The radioreceptorassays (RRA's) can be used to measure placental lactogens derived from many species. Whereas the conventional radioimmunoassay (RIA) is generally species specific, hence RIA has more limited applications. Of course, in any determination the RRA can not distinguish pituitary prolactin (PRL) from placental lactogen or pituitary growth hormone (GH) from placental lactogen (PL). Fortunately, however in most species, serum levels of pituitary prolactin or growth hormone during pregnancy are low as compared to PL 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 pituitary hormone (RIA) concentration represents serum concentration of placental lactogen.

Thus, by employing these two novel radioreceptor-assays (RRA's), several new placental lactogens have been detected in the past two years (Shiu et al,1973; Kelly et al, 1974 a and b; Robertson & Friesen,1974; Fellows et al,1974; Handwerger et al,1974). 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. However, because rat and sheep placental lactogens have been most intensively characterized by the radioreceptorassays, I wish to restrict my discussion to these two hormones only.

Rat Placental Lactogen (rPL)

Chemistry.

Upon purification (Robertson and Friesen,1974), it is found that the molecular weight of rat placental lactogen (rPL) is approximately 18,000 as determined by gel filtration on Sephadex G-100, and 22,000 as determined by electrophoresis sodium dodecyl sulfate (SDS)- acrylamide gels. The isoelectric point of rat placental lactogen is between pH 6.5 and pH 7.0 as determined by isoelectric focusing. Amino acid composition and chemical structure are not known.

Physiology.

Employing radioreceptorassay for prolactin(RRA-PRL), Robertson and Friesen (1974) demonstrated that purified rPL is 41% as active as ovine prolactin standard (NIH-S-P-10, 25 IU/mg) but 169% as active as a human placental lactogen preparation. In the radioreceptorassay for growth hormone (RRA-GH), purified rat placental lactogen has minimal growth promoting potency as compared with bovine growth hormone.

Secretion.

With the receptor assay for prolactin (Shiu et al, 1973), it has been possible to accurately measure serum concentrations of rPL throughout pregnancy. In contrast to the reports of several investigators demonstrating that rat

chorionic mammotropic activity (rCM) or rat placental lactogen (rPL) was maximal in serum and in placental extracts at day 12 of gestation using classical bioassay techniques (Astwood and Greep,1938; Matthies,1974), two peaks of rPL were observed during pregnancy. The highest concentration of rPL occurs on day 12, reaching a mean level of 1584 ± 632 ng/ml (ovine prolactin used as standard), and by day 14 has declined to 180 ± 24 ng/ml (Shiu et al,1973). Further characterization of this secondary peak-activity using gel filtration on Sephadex G-100 (Kelly et al,1973), demonstrated that the molecular weight of rPL in serum at day 20 was approximately 100,000 M.W. (void volume) and 18,000 M.W.. Measurement of prolactin and growth hormone activity by 2 RRA's respectively showed that the material in the void volume (100,000 M.W.) is mainly growth hormone-like by RRA-GH with very little prolactin-like activity by RRA-PRL. In contrast, the 18,000 M.W. material is largely prolactin-like with very little growth hormone-like activity by 2 RRA's. Further, the half-time disappearance rates of rPL from day 12 and 19 were also different. RPL from serum at day 19 was rapid (1.2 min.) compared to day 12 (19.5 min). Although rPL has not been detected in any previous studies at days 17 - 20, the positive existence of such a hormone was suggested by studies of Freinkel and colleagues (Knopp et al, 1970), who observed that rats in late pregnancy exhibited insulin resistance and hyperinsulinemia. Whether rPL detected by RRA's is the hormone mediating these changes remains to

to be proven.

The situation is even more complicated. When rat serum samples during pregnancy are also measured by the radio-receptorassay for growth hormone (RRA-GH)(Kelly et al,1974b), on day 12, the serum rPL has a ratio of prolactin to growth hormone-like activity of 13:1, whereas on day 18 the ratio of PRL/GH changes to 1:1. When placentas were extracted from these two days separately, the ratios of PRL/GH were found to be 110:1 and 45:1,respectively. Thus, it appears that two possible humoral factors might account for the metabolic changes observed in late pregnancy. However, the possibility of interference caused by serum proteins in the radioreceptorassays leading to spurious results cannot be eliminated. Nevertheless, the appearance of double peaks (day 12 and day prior to parturition) of rPL during pregnancy is strikingly different from the secretion pattern of hPL and mPL in human and monkey, respectively. Whether each peak has a different physiological role remains to be defined.

Sheep Placental Lactogen (oPL)

Chemical, Immunological, and Biological Studies -

Upon gel filtration on Sephadex G-100, the molecular weight of oPL appears to be approximately 22,000 (Kelly et al, 1973; Fellows et al,1974; Kelly et al,1974a; Chan et al,1975).

When tested against oPL antiserum, Fellows and coworkers(1974) reported that partially purified oPL shows partial identity to ovine growth hormone (oGH) but there is no cross-reaction with hPL, hPRL, hGH, or ovine prolactin by Ouchterlony diffusion techniques. However, in our laboratory we could not reproduce these results. Handwerger and coworkers (1974) also reported that their partially purified oPL preparation is a potent lactogen which stimulates lactation in vivo in the rabbit intraductal assay and casein synthesis in vitro in mouse mammary gland explants. In the radioreceptorassay for prolactin, oPL displaced the ^{125}I-hGH tracer in a parallel fashion. However, oPL has only 1/6 as much growth hormone binding activity as prolactin binding activity.

Secretion-

Ovine placental lactogen can be detected in plasma samples using radioreceptorassays for prolactin (Kelly et al, 1973). By day 60 of gestation and thereafter placental lactogen concentrations increase as pregnancy advances, reaching peak concentrations of 1,000 to 2,000 ng/ml. 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 1,000 ng/ml to 500 - 700 ng/ml. by 12 hours before parturition; they then decreased quite rapidly post-partum. The relative half-time rate of disappea-

rance of oPL as estimated by ligation of uterine vessels on day 140 of pregnancy appears to be less than 20 min.. When both the foetal and maternal cotyledons were extracted in 0.1 M ammonium bicarbonate, the concentration of oPL in extracts was 200 ug per gram wet weight of tissue which is similar to the concentration of hPL in human placental extracts (Friesen,1965).

By re-assaying the serum samples during pregnancy with radioreceptorassay for growth hormone (RRA-GH), Kelly and co-worker (1974b) reported an interesting observation that the serum concentration of growth hormone-like activity is much lower than prolactin-like activity, the ratio of PRL/GH activity is approximately 3 or 5:1, whereas in placental extracts, the ratio of PRL/GH activity is about 1:1. With the different ratios of prolactin-like to growth hormone-like activities in serum and in placental extracts in this species, in which the corresponding pituitary growth hormone and prolactin activities do not overlap in the RRA's, the possibility that the placenta secretes two hormones must be considered. However, as shown in the subsequent studies to be reported later, purified oPL has these two activities, namely the somatotropic and lactogenic effects.

SECTION III OBJECTIVES OF THE PRESENT INVESTIGATION

The objectives of this investigation were two fold: (1) to purify and characterize sheep placental lactogen(oPL), and (2) to examine some biological effects of ovine placental lactogen.

This investigation describes:

(a) a procedure for the purification of ovine placental lactogen from sheep placental cotyledons in sufficient quantity and sufficient purity for chemical and biological studies, thus firmly establishing the existence of this hormone in sheep, and

(b) comparing the growth promoting effect of ovine placental lactogen, human placental lactogen, and human pituitary growth hormone in receptor assays.

SECTION IV: METHODS OF PURIFICATION AND CHARACTERIZATION

MATERIALS AND METHODS

Assays for monitoring hormonal activities - Radioreceptor-assays for prolactin or lactogen (RRA-PRL) and for growth hormone (RRA-GH).

Radioreceptorassays for measuring prolactin or lactogen utilized rabbit mammary gland receptors as described by Shiu et al (1973) while the one for measuring growth hormone or growth hormone-like activity employed rabbit liver receptors (Tsushima and Friesen,1973) with slight modification.

1. Method of isolation of specific receptors:

Mammary tissue for RRA-PRL or liver for RRA-GH obtained from mid or late pregnant rabbits was cut into small fragments before being homogenized in 5 volumes of 0.3M sucrose solution. Homogenization was carried out at 4 C using Polytron PT-10 (Brinkmann) for one minute with the dial set at 7. The homogenate was filtered twice, first with 4 layers and then through 8 layers of cheesecloth. The filtrate was centrifuged at 780 X g for 20 min. at 4 C, the supernatant was centrifuged at 15,000g for 20 min., and the pellet was discarded. The supernatant was again centrifuged at 100,000g for 90 min. to obtain the total microsomal pellet which contains most of the broken cell membranes. 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 $CaCl_2$, and kept frozen at - 20 C. When required for assay the frozen receptor was thawed, homogenized in a glass homogenizer with 10 mM $CaCl_2$ 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 mammary tissue (about 100 grams) will provide sufficient material for as many as 1,000 determinations, whereas 100 grams of liver tissue provides sufficient receptors for 6,000 GH assays.

2. Iodination procedure for hormone preparations (ovine prolactin(oPRL, NIH-S-P-10) for RRA-PRL and human growth hormone (hGH, NIH-HS 1648E) for RRA-GH) :

^{125}I-oPRL or ^{125}I-hGH was prepared by the lactoperoxidase method of Thorell and Johannson (1971), using 1 mCi of Na^{125}I (New England Nuclear), 5 ug of oPRL or hGH, 4 ug of lactoperoxidase, 2 ul of 30% hydrogen peroxide at 1:1500 dilution, and 25 ul of 0.05M phosphate buffer, pH 7.4 in a final volume of 106 ul.. At the end of one minute chemical reaction time, 1 to 2 ml. of 0.025M Tris-HCl, pH 7.6 was added immediately to the reaction tube after 10 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 0.025M Tris-HCl, pH 7.6 as eluting buffer. The Sephadex G-100 column was pre-treated at once with 1 - 2 ml of 0.025 M 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, 10 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 0.01M phosphate buffered saline, pH 7.4 containing 2.5% 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 780g for 20 min.. The supernatant was decanted and the precipitate was counted in the LKB auto-gamma counter. The incorporation of 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 of radioactivity into oPRL or hGH was approximately 55 -65%, 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 (12 X 75 cm) containing 200 ul of diluent, 100 ul of hormone standard (oPRL or hGH), or 100 ul of known or unknown sample, and 100 ul of ^{125}I-oPRL (approximately 100,000 cpm) or ^{125}I-hGH (approximately 80,000 cpm). During the 6 hours incubation period for the lactogenic assay and or 3 hours for growth hormone at room temperature, the tubes were shaken vigoreously for 30 seconds every 30 min. The reaction was terminated by the addition of 3 ml of ice-cold 0.025M Tris-HCl buffer, pH 7.6 containing 0.1% BSA for lactogenic assay or 0.025M sodium acetate buffer, pH 5.4 containing 0.1% BSA for growth hormone assay. Under these conditions, the hormonal receptors were sedimented by centrifugation at 780g for 20 min. at 4 C. The supernatant was decanted and the membrane bound ^{125}I-oPRL or ^{125}I-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) sheep placental cotyledons removed at the time of surgery were kindly provided by Dr. H. Robertson, Reproductive Physiology, Animal Research Institute, Ottawa, Canada, and Dr. V Chernick, Faculty of Medicine, Dept. of Pediatrics, University of Man., Winnipeg, Canada. These tissues were immediately frozen and stored at - 20 C with or without separating maternal and foetal cotyledons, (2) Sheep placental cotyledons (foetal)

obtained within 1 or 2 hours after parturition were stored frozen at - 20 C. These tissues were kindly provided by Dr. H. Robertson, Reproductive Physiology, Animal Research Institute, Ottawa, Canada, and Dr. N.E. Stanger, Faculty of Agriculture, Dept. of Animal Science, University of Manitoba, Winnipeg, Canada.

Hormone preparations

Human growth hormone (NIH-HS 1648E), ovine prolactin (NIH-P-S-10), bovine growth hormone (NIH-BG B1003A), and all other hormone preparations were kindly supplied by the Endocrine Study Section of the National Institutes of Health, U.S.A.

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 4 C 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 were immediately frozen at -20 C without separating maternal and foetal cotyledons. At the time of extraction, 1.2 kilograms of placental cotyledons (56-65 days gestation) were homogenized with a Polytron PT-10 (Brinkmann) homogenizer at maximum speed for 30-60 seconds in 0.1M ammonium bicarbonate solution, adjusted to pH 9.5 with 1 N ammonium hydroxide, using a ratio of buffer to tissue of 5:1 (v/w). The homgenate was stirred overnight and then centrifuged at 30,000g for 30 minutes. The pellet was discarded.

2. Ammonium sulfate precipitation-

To the supernatant was added slowly ammonium sulfate to a final concentration of 40% saturated solution. After allowing the precipitate to settle overnight, the mixture was centrifuged at 30,000g for 20 min., the precipitate was discarded. To the supernatant was added slowly additional ammonium sulfate to achieve a 75% saturated solution. After stirring the solution for an hour, the precipitate was allowed to settle overnight and subsequently collected by centrifugation.

3. Dialysis-

The precipiate was dissolved with 0.1M ammonium bicarbonate solution (all precipitate dissolved at this time) and dialyzed against running tap water for 48 hours, then dialyzed against distilled water for 48 hours. After dialysis, the solution was centrifuged at 100,000g for 90 min to remove visible particles.

4. Diethylaminoethyl (DEAE) anion exchange chromatography-

To the supernatant, solid ammonium bicarbonate was added to make a 0.05M solution, pH 7.8. The 0.05M supernatant was applied to a column (40 X 60 cm) of diethylaminoethyl cellulose (Whatman DE-32) previously equilibrated with 0.05M ammonium bicarbonate,pH 7.8. After the column was washed with an addtional volume (5 liters) of starting buffer, the concentration of ammonium bicarbonate was increased stepwise from 0.05 to 0.1, and 0.2M. Finally, the column was washed

with 0.5M NaCl in 0.2M bicarbonate buffer. The active fractions were collected, pooled, and concentrated to 50 ml by ultrafiltration using UM-10 membrane.

5. Gel filtration-

The concentrated material was applied to a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (4.2 X 104 cm) which was equilibrated with 0.01M ammonium acetate,pH5.0. Fractions (10 ml/fraction) were collected in a LKB fraction collector.

6. Carboxymethyl-cellulose (CMC) cation exchange chromatography-

Appropriate fractions from Sephadex G-100 column were pooled and applied to a column (1.8 X 18 cm) of carboxymethyl-cellulose (Whatman CM-23) which was also equilibrated with 0.01M ammonium acetate buffer, pH 5.0. After washing the column with 300 ml of starting buffer, a stepwise elution with NaCl at 0.01, 0.05, 0.1, 0.15, and 0.2M was carried out in the presence of 0.01M ammonium acetate, pH 5.0. The column was finally eluted with 0.5M NaCl. The fractions containing oPL were pooled and then concentrated to a volume of 3 ml..

7. Gel filtration-

Finally, the concentrated material was applied to a Sephadex G-100 column (1.4 X 94 cm) which was equilibrated with 0.1M ammonium bicarbonate, pH 8.7. The active fractions were pooled, concentrated to a volume of 1.5 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 acrylamide gel electrophoresis, a 7.2% acrylamide and pH 8.8-9.0 were used, whereas under acidic conditions, 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 to 2 ml of 0.1M Tris-HCl, pH 7.6 containing 0.1% BSA at 4 C for 24 hours with shaking (Fisher Rotator). The eluants subsequently were analyzed by the two radioreceptorassays.

For the alkaline polyacrylamide gel, 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 acid gel polyacrylamide gel, the same procedure was employed except 1% Basic Fuchsin dye was used instead of Amido Black.

(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%. In the final preparation of purified oPL, duplicate samples were run, one gel was stained with 0.1% Coomassie Brillant Blue R-250 containing 3.26% sulphosalicylic acid and 10.86% of trichloroacetic acid (TCA), while the other was cut serially and the individual segments were eluted in 1 to 2 ml. of 0.1M Tris-HCl, pH 7.6 containing 0.1% BSA (w/v) for 24 hours at 4 C. Subsequently the eluants were analyzed by the 2 RRA's.

The staining procedure was 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 and the individual segments were eluted with distilled water for 24 hours at 4 C with shaking (Fisher Rotator). The pH of the eluant was determined by pH electrode (Fisher, Acumet, Model 420).

(c) Preparative isoelectric-focusing-

The technique used was essentially that recommended in the 8100 Ampholine instruction manual. Separations were obtained using an isoelectric focusing column of 110 ml capacity (LKB Ins. Broma, Sweden) and carrier ampholytes in the

range of pH 3.5 ~ 10 obtained from the same source. Gradients were routinely run with the cathode in the upper electrode position. Gradients were prepared in an LKB 8121 gradient mixer and loaded at 1 - 2 ml per minute with a peristaltic pump (LKB,12000 Broma, Sweden). Electrofocusing was allowed to proceed until a constant current was achieved(20~24 hours). The gradient was displaced by the addition of water to the top of the column and 2 ml fractions were collected with the flow rate of 2 ml/min.. Protein concentrations were monitored by absorbance at 278 nm, pH of the fractions were measured by pH electrode (Fisher, Accomet, Model 420), and the oPL activity was determined by the 2 RRA's.

(d) Molecular weight estimation~

The molecular weight of oPL was estimated by gel filtration on a Sephadex G~100 column (2.5 X 75 cm). The column was equilibrated at room temperature with 0.01M Tris~HCl containing 0.1% BSA and 0.1M NaCl at pH 7.6 and was calibrated with ^{125}I~hGH as a marker protein. The hGH was labelled with ^{125}I using the enzymatic method described by Thorell and Johannson (1971).

2. Immunological:

(1) Immunization procedures:

Anti~sera to oPL were raised in New Zealand white rabbits (female, 2~3 kg) obtained from Canadian Breeding

Farm & Laboratory, Canada, using Freund's complete adjuvant. Four rabbits were injected subcutaneously once a week for the first 3 weeks with 200 ug of oPL each in 0.5 ml of 0.1 M ammonium bicarbonate mixed with an equal volume of complete Freund's adjuvant. Thereafter, the animals were injected at 3 week intervals with 50 ug of oPL per animal. The animals were bled after the third injection and the serum was tested for antibodies to oPL.

(2) Iodination of oPL:

^{125}I-oPL was prepared by the chloramine T method of Greenwood, Hunter, and Glover (1962), using 1 mCi of Na^{125}I (New England Nuclear), 5 ug of purified oPL, 100 ug of chloramine T, 250 ug of sodium metabisulphite, 1 mg of potassium iodide, and 250 ul of 0.05M phosphate buffer, pH 7.4 in a final volume of 300 ul. Unreacted iodide and damaged hormone were separated from intact ^{125}I-oPL by gel filtration on a Sephadex G-100 column (1.8 X 45 cm) using 0.01M phosphate buffered saline (PBS), pH 7.4, containing 2.5% BSA (w/v). The percentage of incorporation of radioactivity into oPL was 55-65%. The specific activity of the oPL tracer was 110 - 130 uCi/ug of protein.

(3) Tests for integrity of ^{125}I-oPL:

Fractions eluted from the Sephadex G-100 column were tested for specific binding on rabbit liver receptor assay. The fractions which had the highest specific binding were used in subsequent immunoassays. The specific binding assay

was performed according to the method described by Tsushima & Friesen (1973) except that ^{125}I-oPL was used as tracer, and oPL was used as cold hormone. The specific binding is defined as the difference in radioactivity bound between the tube containing no "cold" hormone and that with excess hormone (2.0 ug/ml.).

(4) Radioimmunoassay procedures:

A double antibody radioimmunoassay (Beck et al,1965) was used. All dilutions were made in 0.01M PBS, pH 7.4 containing 2.5% BSA.

During the assay, approximately 30,000 cpm of ^{125}I-oPL in 0.1 ml. of oPL standard or assay sample, and 0.5 ml of 0.01M PBS, pH 7.4 containing 2.5% BSA. After 72 hours incubation at 4 C, sheep anti-rabbit gamma globulin serum (1:50 dilution) in 0.1 ml was added to the incubation medium, and a further excess of normal rabbit serum (1:30 dilution) in 0.1 ml was added. After another 24 hours incubation period in the cold, the precipitates formed were centrifuged at 780g for 15 min. and the supernatants were decanted. The precipitates were counted in a LKB gamma counter (Model 8000). To test the specificity of the assay, hormone preparations from pituitary, placenta, or pituitary and placental extracts were serially diluted with 0.01 M PBS, pH 7.4 containing 2.5% BSA and added to the assay mixture instead of oPL standards.

3. Biological:

(a) Bioassay of growth promoting activity-

Female Sprague-Dawley rats were obtained from Canadian Breeding Farm & Laboratory Ltd, Montreal, Canada. All rats were hypophysectomized at 2 weeks of age and kept in rooms under closely regulated conditions of temperature and humidity before and during the experiments. At the end of a 4 weeks acclimatization period, animals exhibiting inappropriate weight gain were discarded. Purified oPL or bovine growth hormone were prepared for injection using equal volumes of chilled saline (0.9% NaCl) and 0.05M 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 in 0.5 ml subcutaneously daily for a period of 9 days using 10 hypophysectomized rats at each dose. The animals were weighed daily prior to the injection. The weight gain of individual rats was calculated and final results were expressed as the mean for each treated group. The potency estimates of oPL using bGH (0.9 U/mg) as standard were calculated according to the method of Pugsley (1946). At the end of the experiment, all rats were killed and the sella turcica of each rat was inspected for completeness of hypophysectomy.

(b) Lactogenic bioassay.

The lactogenic property of oPL was assessed by its ability to stimulate ^{3}H-casein synthesis in rabbit mammary explants maintained in organ culture. The procedures employed were similar to those of Juergens et al (1965) for mouse tissue with slight modification.

Virgin New Zealand white rabbit (2-3 kg.) was made pseudopregnant by a single intravenous injection of 100 U hCG. 12 days later, mammary glands were removed under aseptic conditions and cut into explants about 1 mg each. Four explants were placed on a siliconized lens paper which was floated on 1 ml of culture medium in a Falcon culture dish. The culture medium was Medium 199 (Gibco) which was supplemented with insulin and hydrocortisone, 10 ug/ml each, penicillin and streptomycin at concentrations of 50 ug/ml, and HEPES buffer (10 mM). The dishes were placed in a plastic box (35X 25X15cm) and exposed to 95% air-5% CO_2 sufficient to maintain the pH at about 7.4, after which the system was closed and incubated at 37 C for 72 hours. At the end of 72 hours, explants were transferred into new medium which contained either ovine prolactin (NIH-P-S-10) or a highly purified preparation of oPL at a final concentration of 1 ug/ml. At the end of an additional 24 hours incubation, ^{3}H-leucine was added to the medium such that one ml. of medium contains 5 uCi of ^{3}H-leucine. Incubation in the presence of radioisotope was carried on for an additional 4 hours.

Casein assay. At the end of 4 hours incubation, the explants from each dish were weighed and homogenized in 7 ml. of solution with the following composition: KCl,0.15M; sodium phosphate (NaH_2PO_4), 0.004M; imidazole, 0.01M; Hammarston bovine casein (Nutritional Biochemicals), 5 mg. The final pH was 6.7. The homogenate was centrifuged at 100,000g for 60 min., 5 ml of the supernatant fluid was made 0.01M with respect to $CaCl_2$, and 50 ug of crystalline rennin (Sigma) was added, followed by incubation with shaking at 37 C for 30 min., This procedure resulted in the precipitation of casein. After centrifugation at 780g for 10 min. at room temperature, the precipitate was washed twice with the homogenizing solution (without casein) made 0.01M with respect to $CaCl_2$. The pellet was then heated in 5% TCA at 95 C for 15 min., After cooling, the suspension was centrifuged at 780g for 10 min. at 4 C, washed twice with cold TCA, and three times with absolute alcohol-ether (3:1,v/v) at room temperature. The pellet was dissolved in 0.5 ml protosol. After the addition of 0.1 ml distilled water,the dissolved pellet was counted in toluene scintillation counter (Isocap/300, Nuclear Chicago).

The results are expressed as cpm/mg wet weight of explants.Using this procedure isotopically labeled phosphoproteins (casein) in the 100,000g supernatant fraction are precipitated along with the carrier bovine casein by calcium ions and rennin.

4. Receptor assays:

(a) Displacement curve of oPL in the radioreceptorassay for prolactin (RRA-PRL) and for growth hormone (RRA-GH) using rabbit mammary and liver respectively-

A highly purified oPL preparation was accurately weighed and dissolved in 0.1M ammonium bicarbonate, pH 8.7. Serial dilutions of oPL were made in 0.025M Tris-HCl, pH 7.6 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 & Friesen (1973) as described previously except that ^{125}I-bGH and bGH were used as tracer and standard respectively.

^{125}I-bGH 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 4.2 instead of pH 7.4. 5 ug of lactoperoxidase, 10 ul of 30% hydrogen peroxide (1:1,500 dilution), and reaction periods of 20 min. were used. The percentage of radioactivity incorporated was 55-60, and the specific activity was 110-130uCi/ug of protein.

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

Human livers obtained at autopsy and stored frozen at -20 C were kindly provided by the Health Science Center,

Dept, of Pathology. Human liver receptors were prepared according to the method of Tsushima & Friesen (1973) with modifications described by Carr et al (1975). A 15,000g pellet was used as receptor source instead of the 100,000g microsomal pellet. ^{125}I-hGH and hGH were used as tracer and standard respectively.

SECTION V : RESULTS

Radioreceptorassays for prolactin (RRA-PRL) and for growth hormone (RRA-GH) using rabbit mammary tissue and liver respectively.

The sensitivity of both radioreceptorassays (RRA-PRL and RRA-GH) employed to monitor the hormonal activities is about 10 ng/ml (1 ng) as shown in Figure 2, a and b. In the RRA-PRL, ovine prolactin as well as prolactin of other species, human growth hormone, and primate placental lactogens inhibited the binding of ^{125}I-oPRL to rabbit mammary tissue prolactin receptors, whereas other polypeptide hormones did not inhibit the binding of ^{125}-oPRL. Only human growth hormone but not non-primate pituitary growth hormones competed for the prolactin binding sites. In addition, sheep placental extract inhibited the binding of ^{125}I-oPRL in a parallel manner as shown in Figure 2 a. In the RRA-GH, hGH, growth hormone preparations from other species, and primate placental lactogens inhibited the binding of ^{125}I-hGH to rabbit liver membrane GH-receptor sites in a parallel manner, whereas other hormone preparations do not inhibit the binding of ^{125}I-hGH. Again, sheep placental extract displaced ^{125}I-hGH in a parallel manner with the standard as shown in Figure 2b.

Purification:

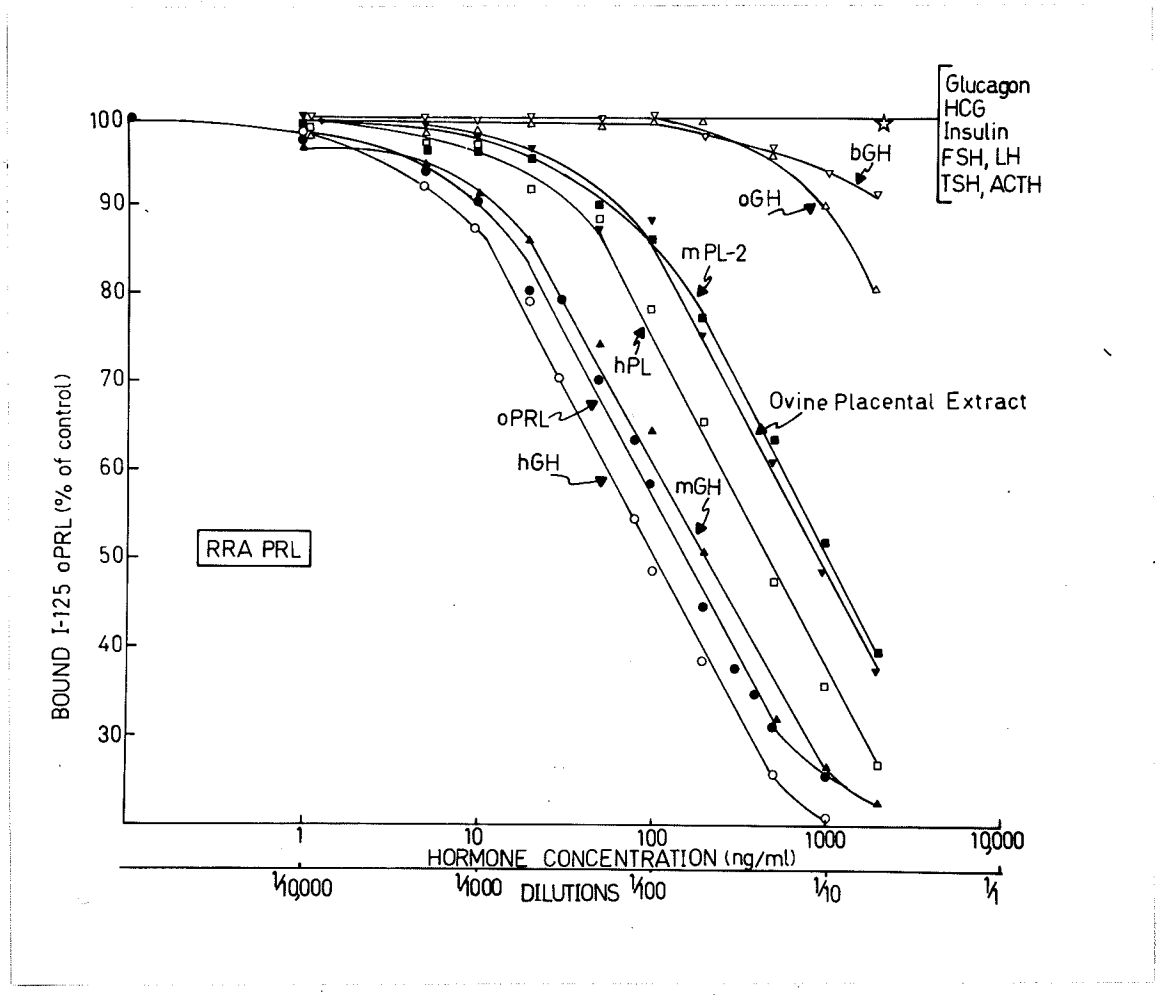

Figure 2a. Radioreceptorassay for prolactin using particulate fraction derived from rabbit mammary glands. The sensitivity and specificity of the binding of the assay is illustrated (from Shiu et al,1973).

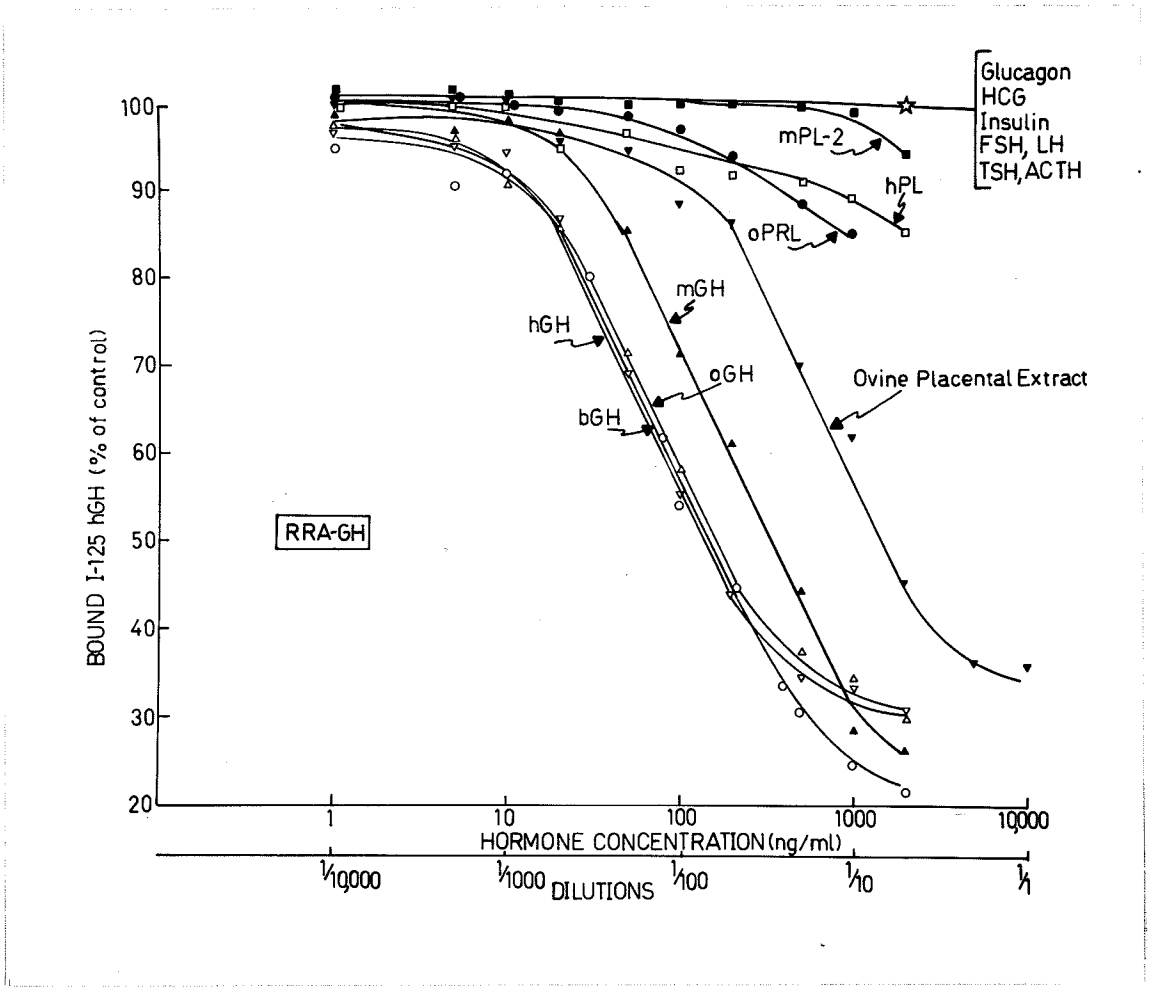

Figure 2b. Radioreceptorassay for growth hormone using particulate fractions derived from rabbit liver. The sensitivity and specificity of binding of the assay is illustrated (from Tsushima and Friesen, 1973)

OPL concentration in placental tissues at different gestational periods.

Figure 3 shows the oPL content of the different starting materials that we have examined. The placental cotyledons at 74 days of gestation and afterward appear to have maximal concentration of oPL. Fresh placental cotyledons obtained at surgery and stored frozen at -20 C were a far richer source of oPL than placental cotyledons obtained after delivery. Although oPL concentration in placental tissues obtained after parturition is low, its content is still sufficiently high (32 ug of RRA-PRL and 22 ug of RRA-GH per gram of wet tissue) to make it a useful alternative when fresh placental tissues (74 days to 145 days of gestation) are not readily available.

Extraction of oPL from frozen placental cotyledons

Several conditions were tested to determine the best procedure to use in the initial extraction of oPL from frozen placental cotyledons. As shown in Table I, by far the greatest amount of oPL was extracted at pH 9.5. Acidic solutions were much less effective in solubilizing oPL. Attempts to recover oPL by re-extraction of the acidic precipitate in 0.1M ammonium bicarbonate, pH 9.5 were unsucessful, less than 1% of oPL could be recovered. Thus, it appears important that the primary extraction should be carried out at an alkaline pH at 4 C.

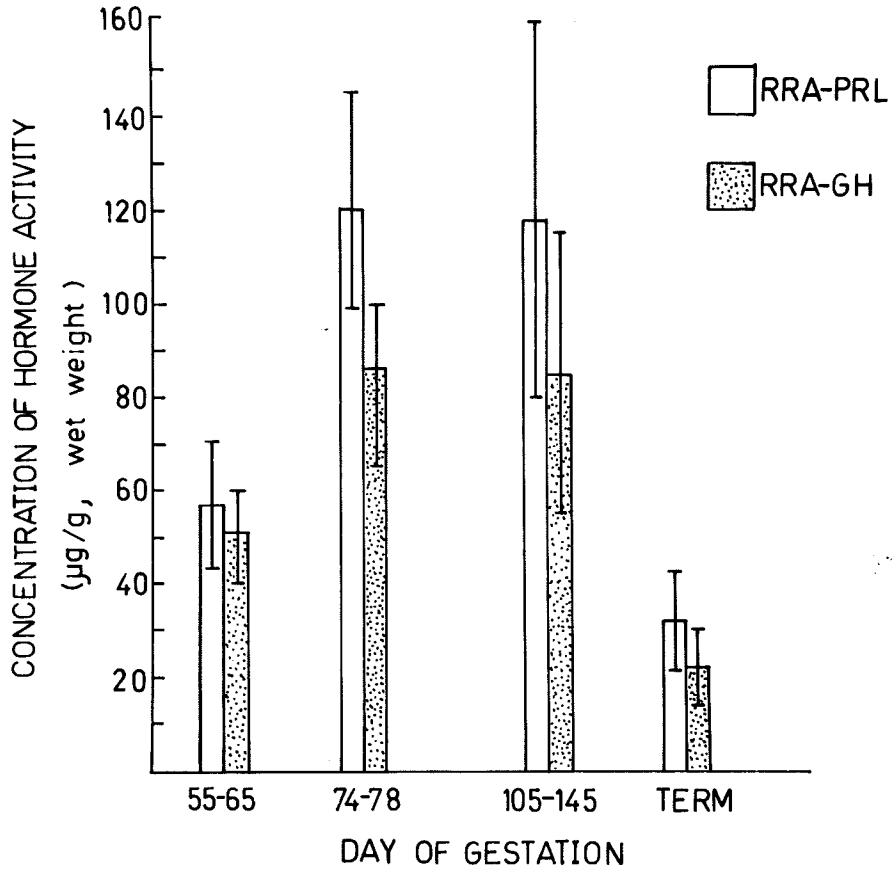

Figure 3. OPL concentration in placental tissues at different gestational periods.Placental cotyledons were homogenized in 5 volume of 0.1M NH_4HCO_3 buffer, pH 9.5 and stirred overnight at 4 C.

TABLE I

EXTRACTION OF OPL FROM FROZEN PLACENTAL COTYLEDONS

Placental cotyledons were homogenized in 5 volumes of extracting fluid and stirred overnight at 4 C.

EXTRACTING FLUID	pH	RRA-PRL (ug/g wet weight)	RRA-GH (ug/g wet tissue)
0.1N NaOH*	10.5	50	45
0.1M NH_4HCO_3 + 1M NH_4OH	9.5	90	75
0.1M NH_4HCO_3 + 1M NH_4OH	8.7	80	68
0.1M Tris-HCl	7.4	47	30
0.1M Ammonium acetate	6.8	2	1.5
0.1M Ammonium acetate + acetic acid**	5.0	<0.1	<0.1
0.1N Acetic acid	3.0	<0.1	<0.1

*pH lowered to 10.5 with 5N HCl ; **pH lowered to 5.0 with 1N acetic aicd.

Fractionation of oPL from crude extracts

after the primary extraction, several fractionation methods were tested to determine the best procedure to use for separating oPL from other proteins present in the crude extract. As shown in Figure 4, it appears that fractional precipitation with organic solvents,such as ethanol(Fig.4a) and acetone (Figure 4 b) were not effective due to the loss of oPL activity during precipitation. Fractional precipitation by lowering the pH of the extract (Fig. 4 c) was also not very effective, since the loss of oPL activity also was high. The best fractional precipitation method for oPL appears to be ammonium sulfate precipitation, as shown in Figure 4 d. Fractions obtained between 40 and 75 percent saturation of ammonium sulfate contain about 60 to 80% of the original oPL activity but only 15 - 25 % of the original protein.

Diethylaminoethyl (DEAE)-cellulose anion exchange chromatography

Figure 5 shows the elution pattern when the dialyzed material after ammonium sulfate precipitation was subjected to DEAE ion exchange chromatography. Most of the oPL was unabsorbed by the column. With increasing ammonium bicarbonate concentrations very little additional hormone was eluted.

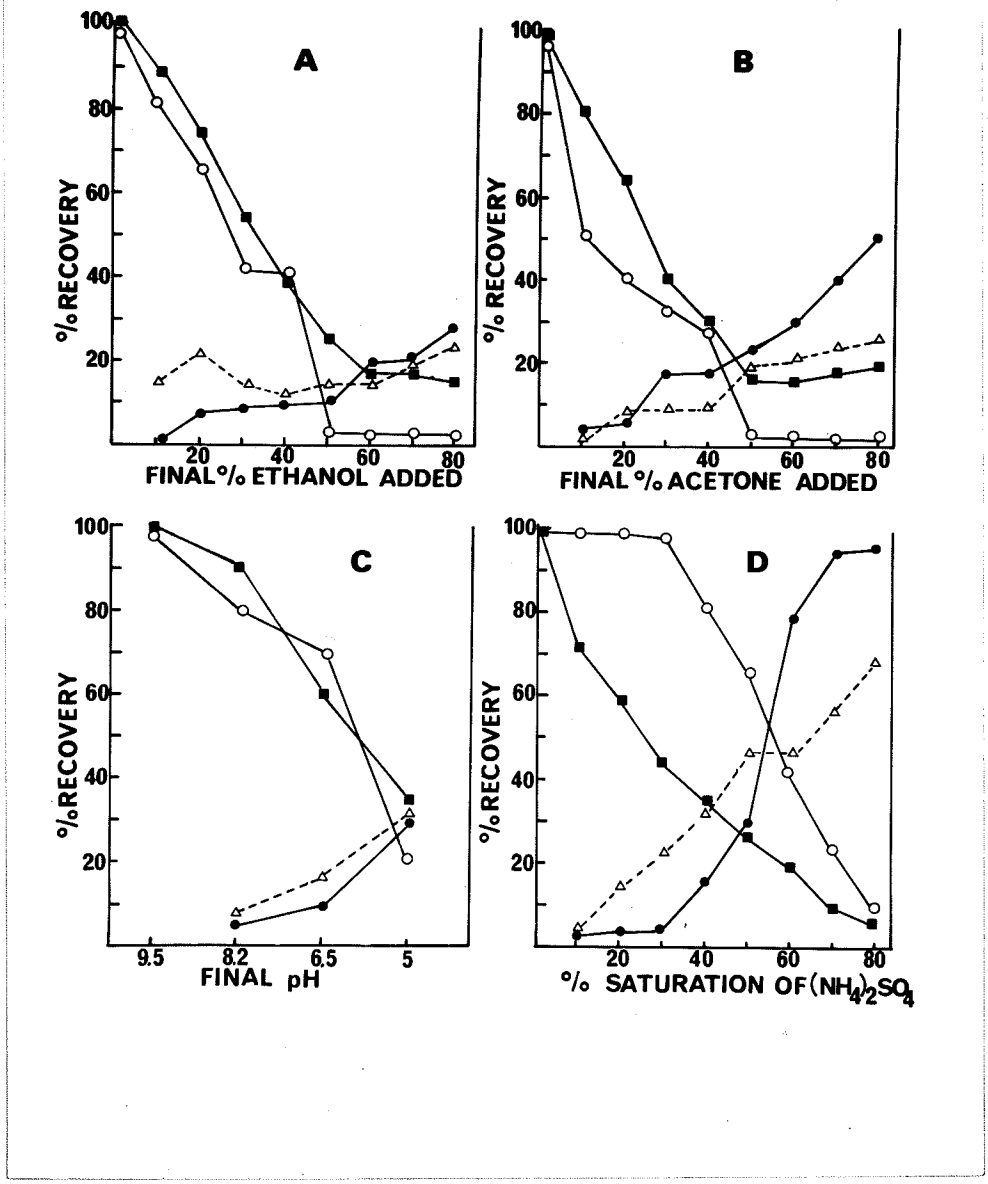

Figure 4. Fractionation of oPL from crude extract. (A) Ethanol. (B) Acetone. (C) pH with 1N acetic acid. And (D) Ammonium sulfate precipitation. % recovery of protein by Lowry ■—■—■—■, and RRA-GH o—o—o—o from supernatant. % recovery of protein ▪—•—•—▪, and RRA-GH Δ—Δ—Δ—Δ from precipitate (soluble protein). Similar pattern of RRA-PRL was obtained (not shown).

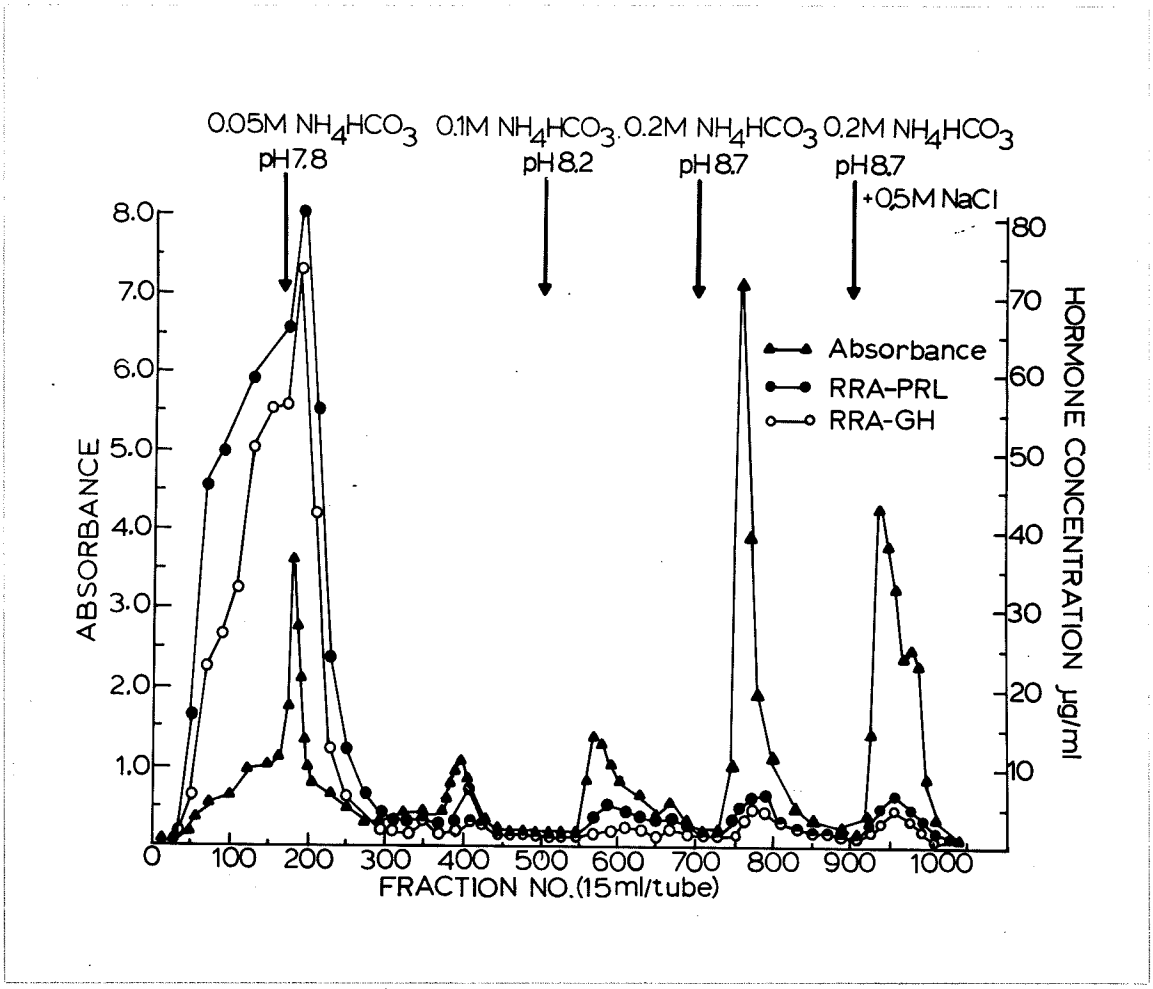

Figure 5. Diethylaminoethyl (DEAE)-cellulose chromatography of oPL rich fraction, the ammonium sulfate precipitate obtained between 40-75% saturation. The DEAE-cellulose (Whatman DE-32) column (60X40 cm) was equilibrated with 0.05M ammonium bicarbonate, pH 7.8.

Gel filtration

Figure 6 shows the distribution of protein and oPL after gel filtration on Sephadex G-100 of the active fractions obtained from the DEAE column. Most of the oPL emerged in fractions with an elution volume of 1.9-2.4 times that of the void volume.

Carboxymethyl (CM)- cellulose cation exchange chromatography

The fractions from the Sephadex G-100 gel filtration were pooled and applied to a CM-cellulose ion exchange column for additional purification, as shown in Figure 7. Most of the oPL was eluted in the presence of 0.2M NaCl, although a small additional peak eluted with 0.5M NaCl. However, analysis upon polyacrylamide gel electrophoresis, indicated that this fraction did not differ from the major peak eluted with 0.2 M NaCl.

Column chromatography

The fractions containing oPL from CMC-chromatography were pooled, concentrated and further purified by gel filtration on Sephadex G-100, as shown in Figure 8. Most proteins including oPL eluted at 1.9-2.4 times the void volume.

A summary of the purification procedure and the recovery of oPL appears in Table II.

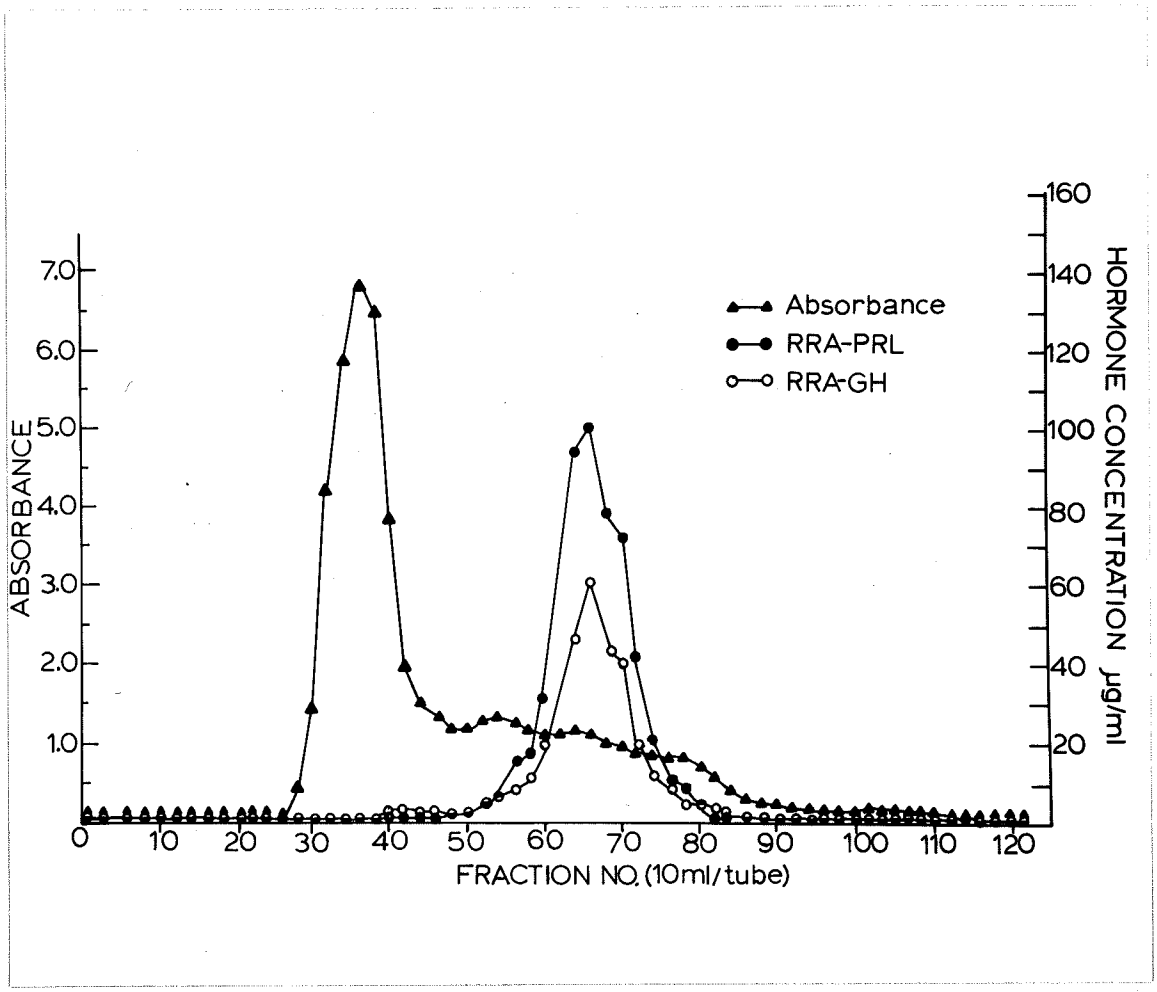

Figure 6. Gel filtration of the oPL rich fractions obtained from DEAE-cellulose column in Figure 5 on a Sephadex G-100 column (104 X 4.2 cm) using 0.01M ammonium acetate, pH 5.0 as buffer.

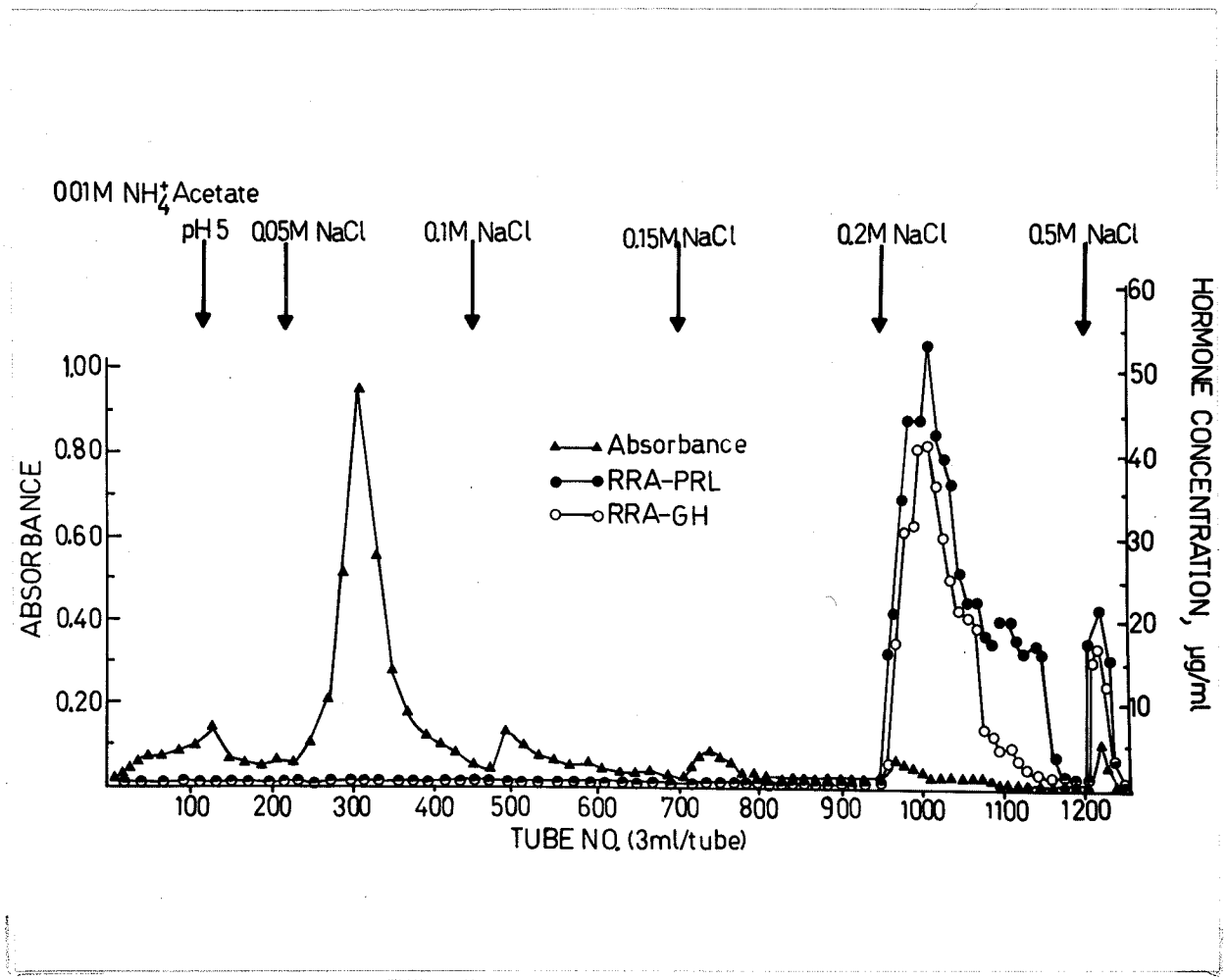

Figure 7. CM- cellulose chromatography of the oPL rich fractions from the Sephadex G-100 column in figure 6 (fractions #56-80). The CM-cellulose (Whatman CM-23) column(18X1.8cm) previously equilibrated with 0.01M ammonium acetate buffer, pH 5.0.

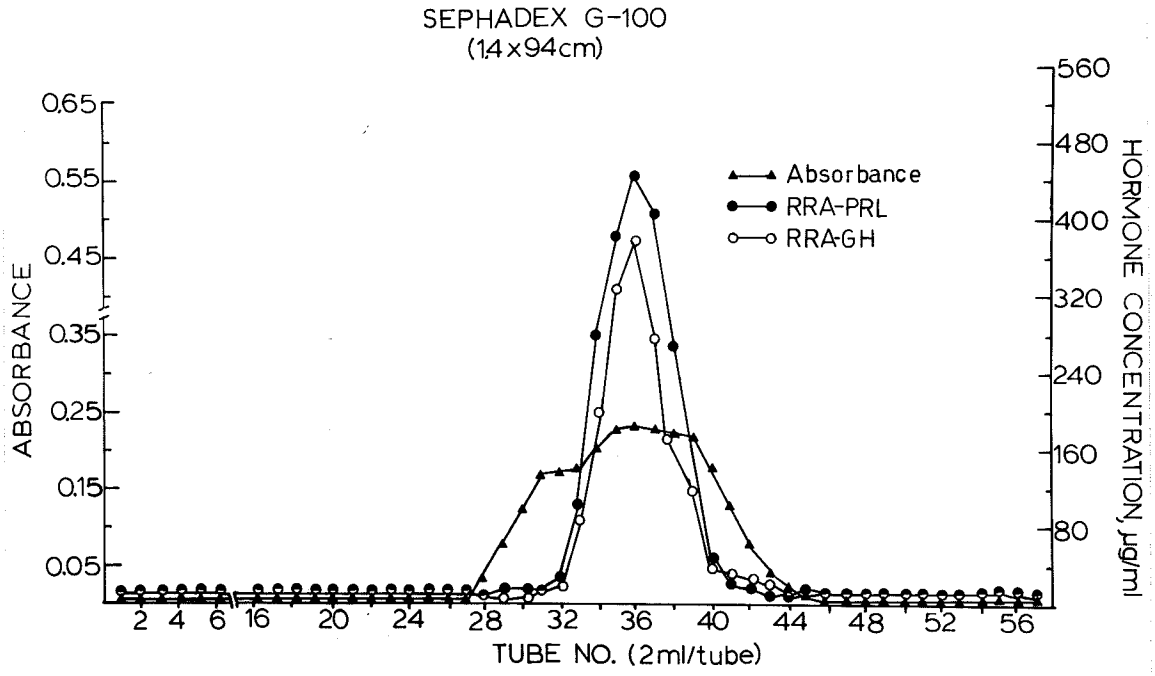

Figure 8. Gel filtration on Sephadex G-100 column (94X1.4 cm) of fractions from the CM-cellulose column in Figure 7. The Sephadex G-100 column was equilibrated with 0.1M ammonium bicarbonate, pH 8.7.

TABLE II

TABLE OF PURIFICATION

PROCEDURE	PROTEIN (g)	RRA-PRL (mg)	RRA-GH (mg)	RECOVERY IN PERCENTAGE			PURIFICATION
				PROTEIN	RRA-PRL	RRA-GH	
Extraction ($0.1M\ NH_4HCO_3$)	61.2	60.8	50.5	100	100	100	1
40-75 % $(NH_4)_2SO_4$	10.6	42.4	33.9	16	70	67	4
DEAE-Cellulose	0.6625	18.8	15.6	1	31	31	29
Sephadex G-100	0.255	18.2	14.2	0.41	30	28	70
CM-Cellulose	---*	10.8	8.4	---	18	16	---
Sephadex G-100	0.0061**	6.0	5.1	0.001	10	10	1001

* not measurable - below the sensitivity of Lowry (50 ug/ml)

** Dry weight

N.B. This procedure was employed for the purification of oPL using 6 separate batches of placenta yielding of a total of 25 mg of oPL. In general, the results were similar to the data shown on this table.

Characterization:

Analytical polyacrylamide gel electrophoresis

Figure 9 shows the protein pattern of the most highly purified oPL preparation upon electrophoresis run under alkaline (pH8.8- 9.0) and acidic (pH 4.3-4.5) conditions . Under the alkaline conditions, ovine pituitary growth hormone and prolactin were also run at the same time for the purpose of comparing their relative mobility (R_f). The R_f values observed were 0.18, 0.27, and 0.6 for oPL, oGH, and oPRL respectively. In the most highly purified preparation of oPL, 3 bands are still evident. When individual gel segments were eluted and assayed for oPL by 2 RRA's, only the middle band was active. Furthermore, when the purified oPL was subjected to polyacrylamide gel electrophoresis at an acid pH, 3 bands again were visible. Once more only the middle band was positive for oPL when eluted segments were assayed as shown in Figure 10.

Analytical gel isoelectric-focusing

The protein pattern upon gel isoelectric focusing at each of the steps of the purification is shown in Fig.11. The highly purified preparation of oPL displayed a number of bands when analyzed by this very sensitive technique. When the eluted segments were assayed as shown in Figure 12 by the 2 RRA's, oPL activity was found to coincide with the

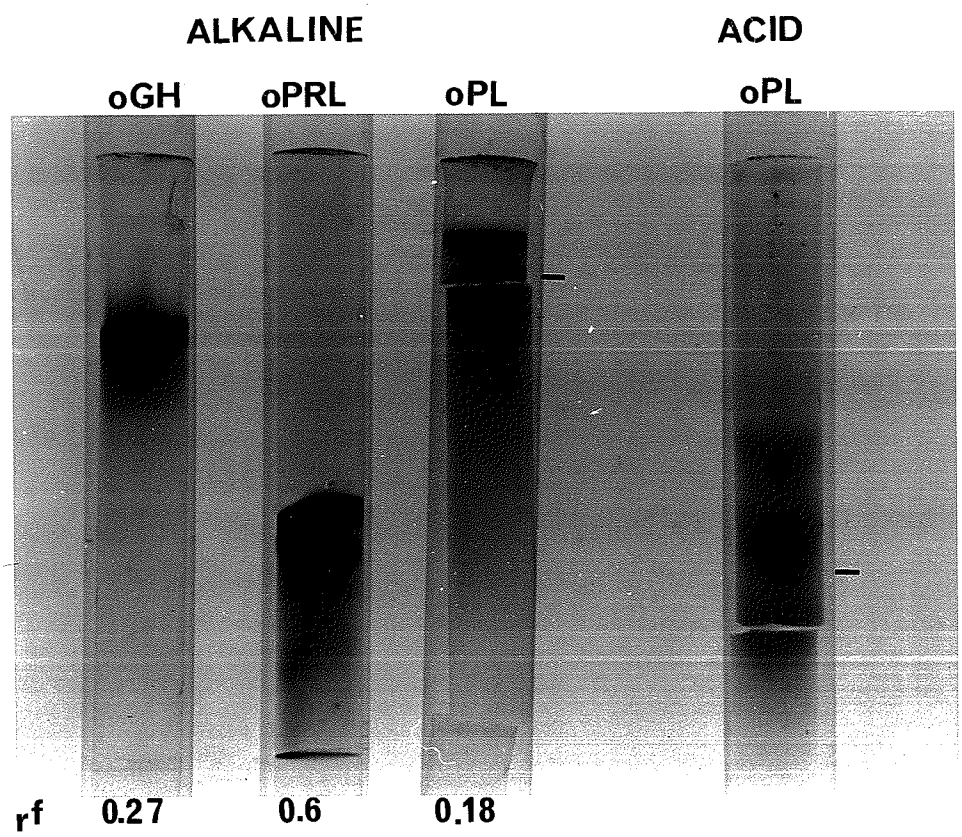

Figure 9. Polyacrylamide gel electrophoresis pattern of oPL. The first three gels represent oGH,oPRL, and oPL run upon alkaline condition at pH 8.8-9.0. The R_f for these 3 hormones are 0.27, 0.6 and 0.18 respectively. The fourth gel represents oPL run under acidic condition at pH 4.3-4.5. The dot beside the two oPL gels indicates the position where the oPL activity is detected by the 2 RRA's.

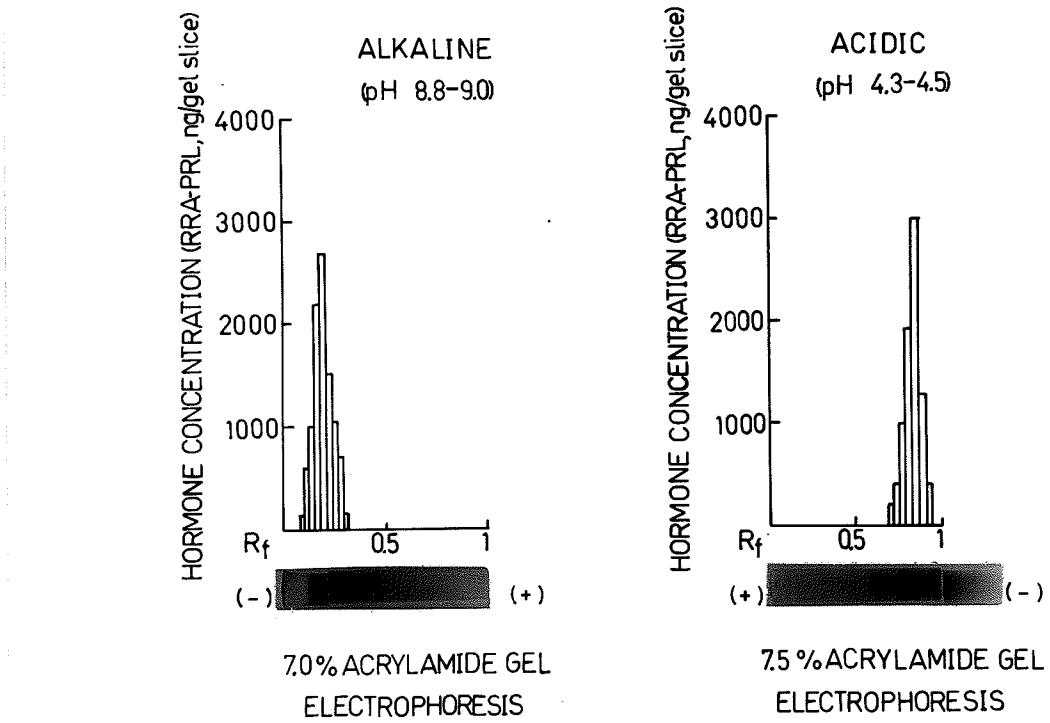

Figure 10. Pattern of oPL activity eluted from polyacrylamide gel electrophoresis. One gel each run under alkaline or acidic condition was segmented at 1 mm intervals and eluted with 2 ml. of 0.1M Tris-HCl buffer, pH 7.6 containing 0.1% BSA. Gel eluants were assayed and the distribution of oPL(shown) was measured by RRA-PRL. Similar pattern was obtained (not shown) by RRA-GH.

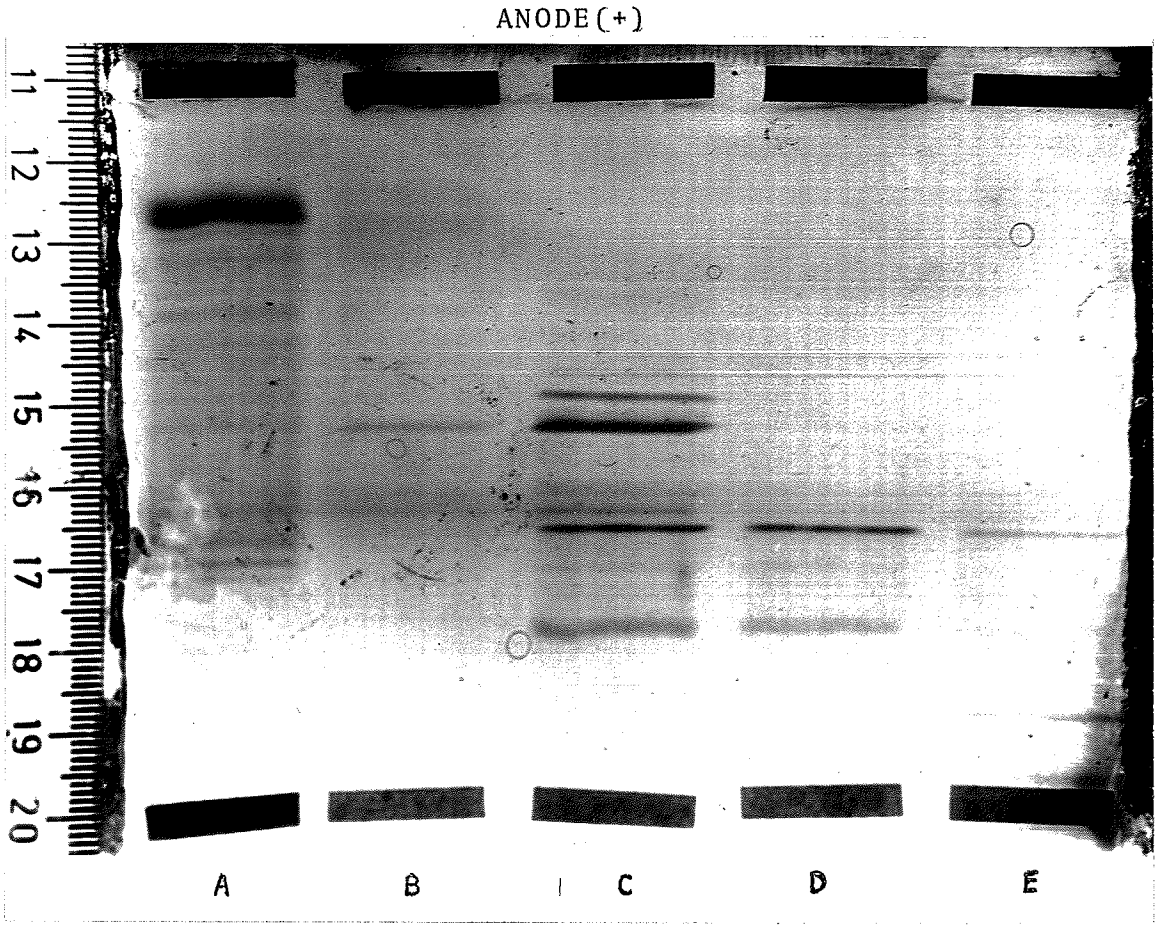

Figure 11. Analytical polyacrylamide gel isoelectric focusing pattern of oPL. The five gels in channels A to E are the fractions obtained during different stages of purification. (A) crude extract, 65 ug dry weight. (B) fraction obtained after Sephadex G-100 chromatography, 50 ug dry weight. (C) fraction obtained after DEAE-cellulose chromatography, 66 ug dry weight. (D) 21 ug and (E) 26 ug are fractions of different batches which were obtained after CM-cellulose and Sephadex G-100 chromatography. Fraction E was used for analysis and characterization of oPL. The anode was placed on the top of the gel, while the cathode was placed near the bottom of the gel.

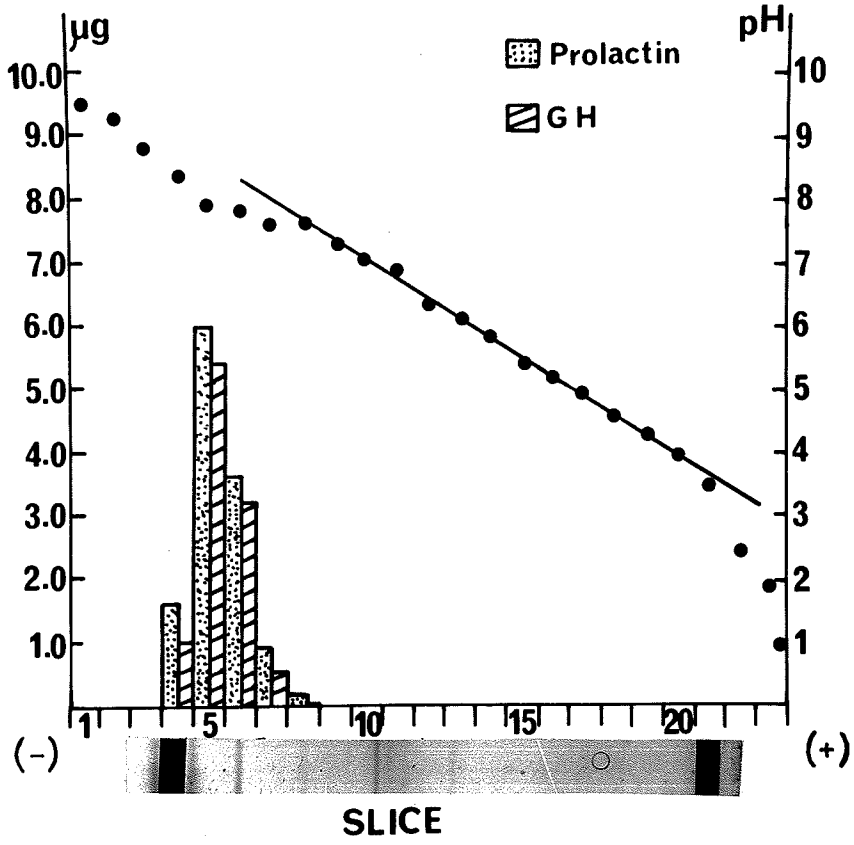

Figure 12. Pattern of oPL activity eluted from polyacrylamide gel isoelectric focusing. The cathode was placed near segment #4, while the anode was placed on segment 21. Gel eluants were assayed, and the distribution of oPL activity was determined by the 2 RRA's. The concentration of prolactin-like and growth hormone-like activities were measured by the RRA-PRL and RRA-GH respectively. These 2 activities were indicated in the same gel segment.

2 bands near the cathode. In addition, when serial dilutions of the eluates from these segments were made, the response curves observed in the 2 RRA's showed complete parallelism to that of ovine prolactin and hGH standard. However, the band which was situated nearest the cathode (paper filter) possesses the highest oPL activity, suggesting that the oPL activity detected corresponding to the other band may be due to incomplete isoelectric focusing.

Preparative isoelectric focusing

Figure 13 shows that when the partially purified oPL was subjected to preparative isoelectric focusing, several regions of UV absorbing material were present but oPL was concentrated in the pH region 8.5-9.0.

Molecular weight estimation

Figure 14 shows that when purified oPL was applied to a Sephadex G-100 column together with ^{125}I-hGH, oPL emerged in the same fractions as ^{125}I-hGH, suggesting that the molecular weights of oPL and hGH are very similar 20,000-22,000 M.W..

Radioimmunoassay for oPL

Figure 15 shows the sensitivity and specificity of the radioimmunoassay for oPL using a double antibody technique. Sheep pituitary growth hormone and prolactin do not

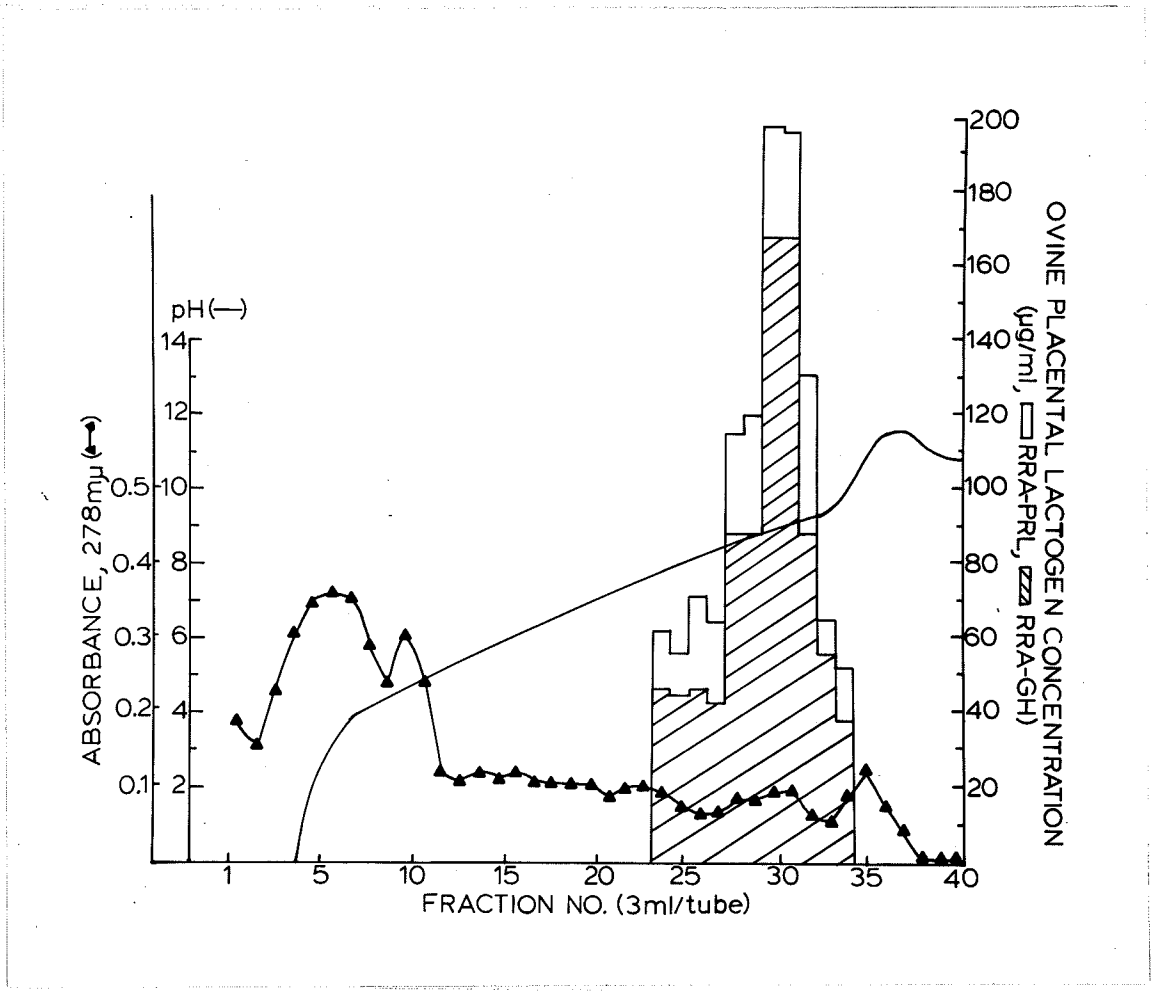

Figure 13. The elution profile of oPL after isoelectric-focusing. Isoelectric focusing was performed in a sucrose gradient containing 1% carrier ampholytes in the pH 3 - 10 range.

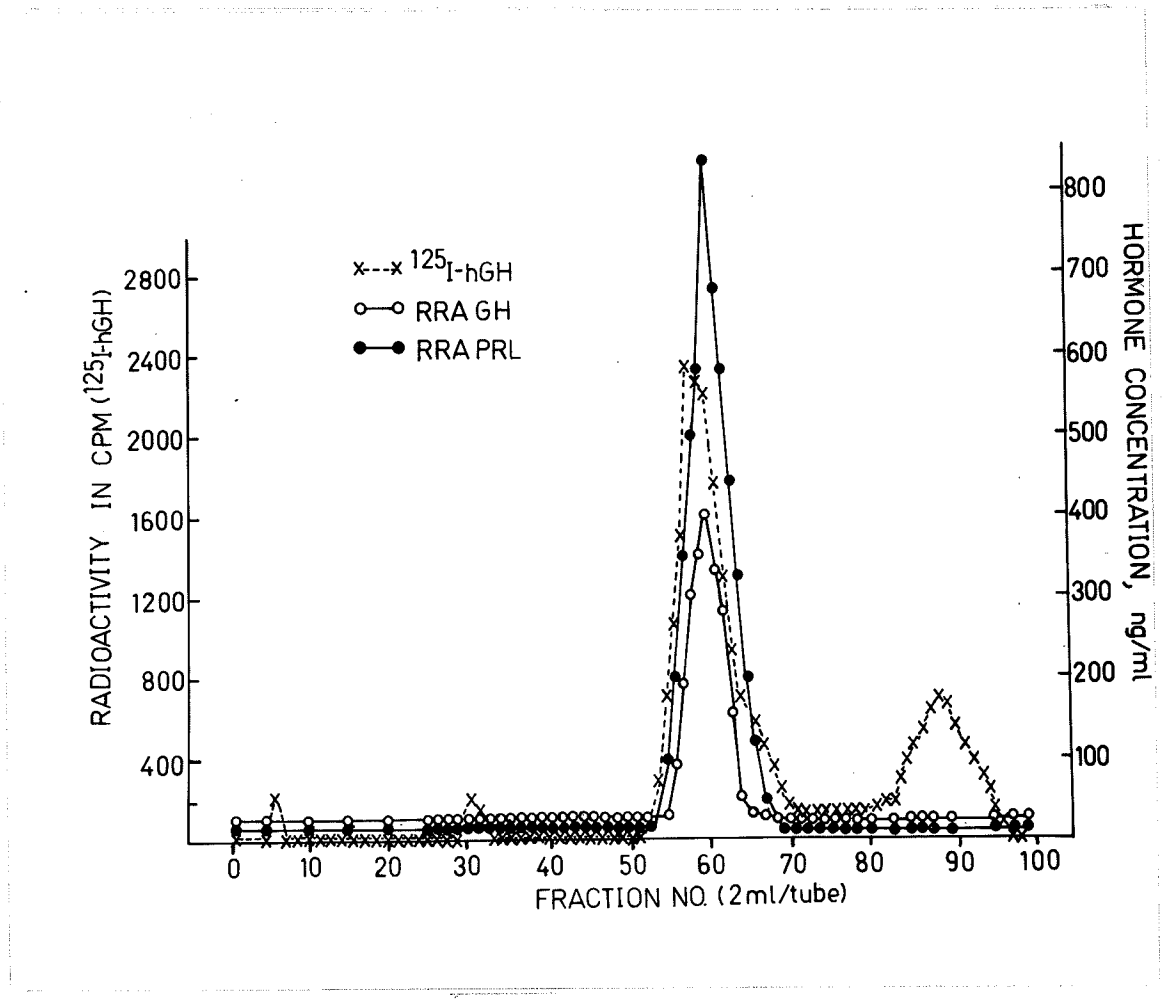

Figure 14. Molecular weight determination of oPL by gel filtration on Sephadex G-100. Sephadex G-100 column(75X2.5cm) was equilibrated at room temperature with 0.01M Tris-HCl containing 0.1% BSA and 0.1M NaCl at pH 7.6 and was calibrated with ^{125}I-hGH as a marker protein.

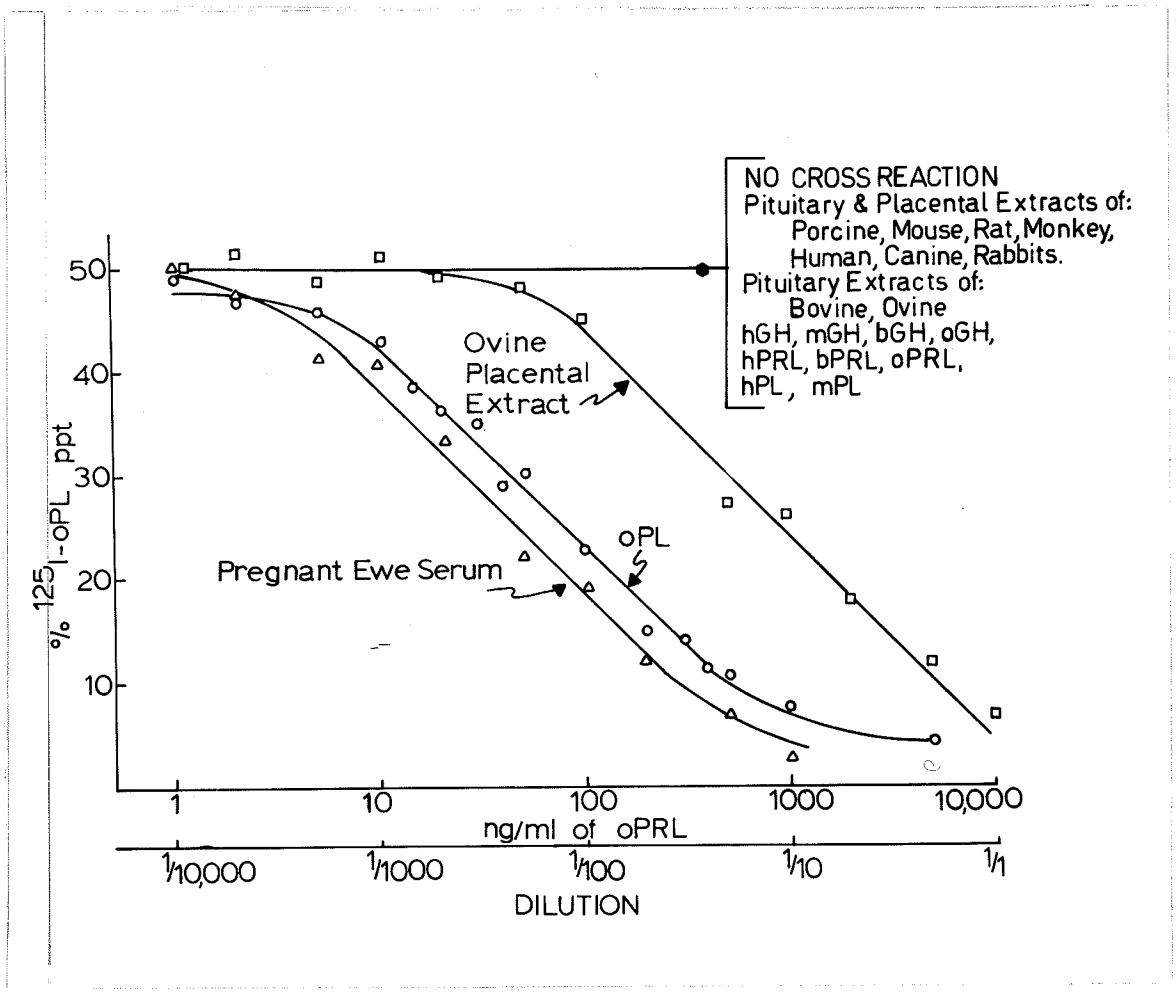

Figure 15. Radioimmunoassay for oPL. Double antibody technique was used for the radioimmunoassay of oPL. To test the specificity of the assay, hormone preparations from pituitary, from placenta, or pituitary and placental extracts were serially diluted with 0.01M PBS, pH 7.4 containing 2.5% BSA and added into the assay.

cross-react with antisera to oPL. Similarly, several other hormone preparations from other species either of pituitary origin or placental origin, showed no cross-reaction at all. The only samples which do cross-react in the assay are sheep placental extract and pregnant sheep serum (129-131 days gestation). These results indicate that oPL is immunologically different from sheep pituitary GH or PRL and also from any other hormone preparation tested.

Bioassay of growth promoting activity

Figure 16 shows the results of the bioassay for growth promoting activity of purified oPL. Purified oPL is 1.5 times more potent than bGH standard (0.9 U/mg). The relative growth promoting potency of oPL is calculated to be 1.3 U/mg with 95% confidence limits of 0.9-1.6 U/mg.

Lactogenic bioassay

Figure 17 shows the results of the bioassay for lactogenic activity of purified oPL. Purified oPL is equipotent with the ovine prolactin standard (NIH-P-S-10,26IU/mg), adding further evidence that oPL is a very potent lactogenic as well as growth promoting hormone.

Displacement curve of oPL in the radioreceptorassay for prolactin (RRA-PRL) and for growth hormone (RRA-GH) using rabbit tissue receptors.

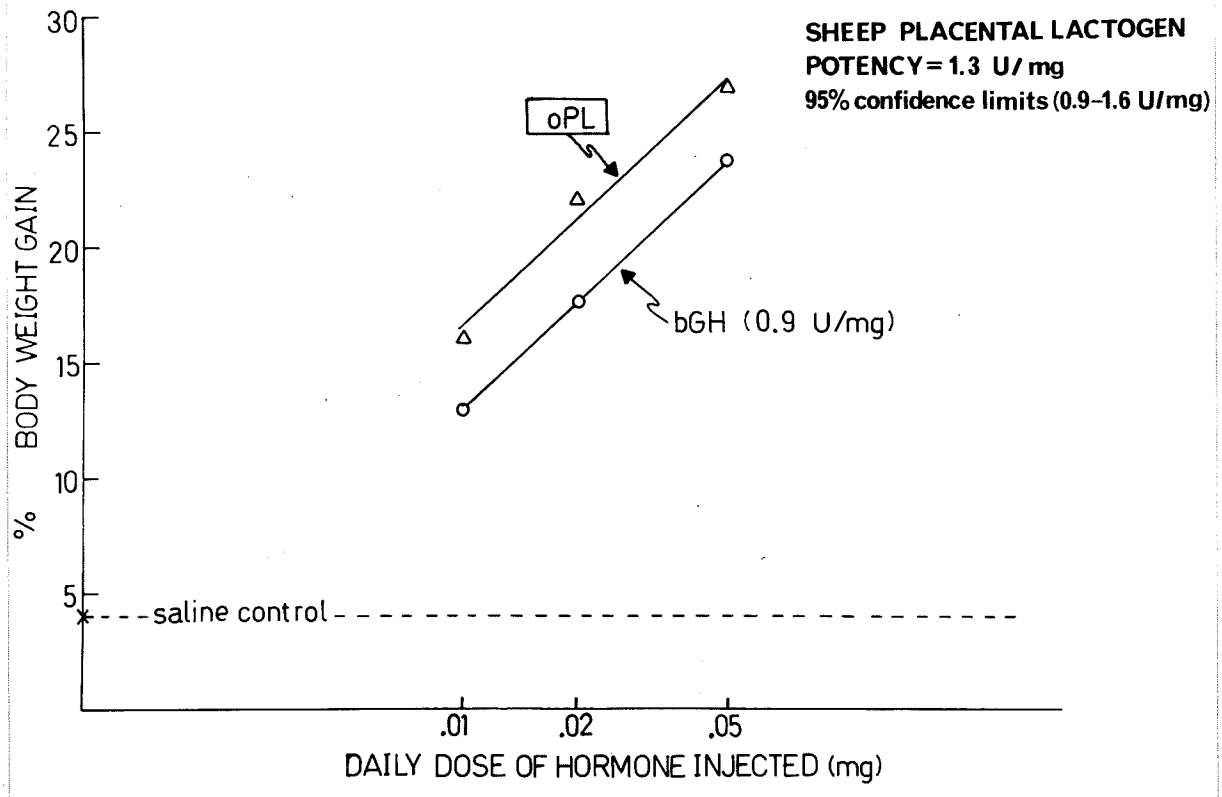

Figure 16. Bioassay of growth promoting activity of oPL. 10 hypophysectomized rats were used at each dose. Bovine growth hormone (0.9 U/mg) was used as standard, and saline was used as control group.

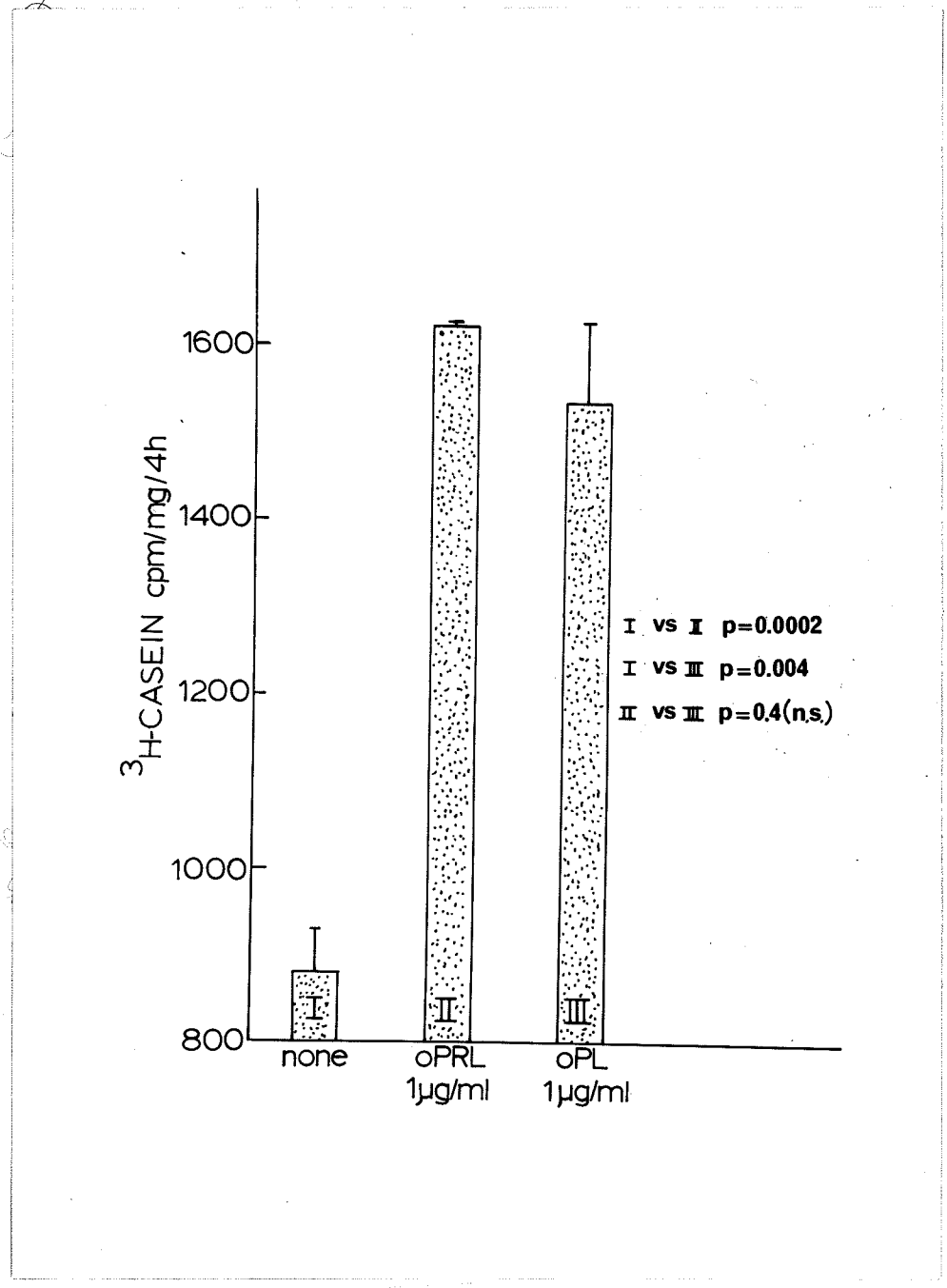

Figure 17. Bioassay of lactogenic activity using organ co-culture of rabbit mammary explants. Results are expressed as cpm/mg wet weight of explants. Each group contains of 3 dishes. Each dish contains of 4 explants. Ovine prolactin was used as standard, and saline was used as control.

When the highly purified oPL preparation was assayed in both RRA's (Figure 18), oPL inhibited the binding of either ^{125}I-oPRL (Fig. 18a) or ^{125}I-bGH (Fig. 18b) in a parallel manner to the hormone standards used (oPRL for RRA-PRL and bGH for RRA-GH respectively). 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 was apparent that oPL has a ratio of 2:1, whereas the ratio of hGH is 1:1 and of hPL is 100:1.

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

In human liver receptor assays, oPL was able to displace ^{125}I-hGH from its binding sites as effectively as hGH (Figure 19), whereas hormone preparations from other species showed no competition for binding sites. Only primate pituitary growth hormone and ovine placental lactogen compete for binding sites in the assay. Admittedly hPL showed very minimal cross-reaction in the assay in keeping with its weak growth promoting effect.

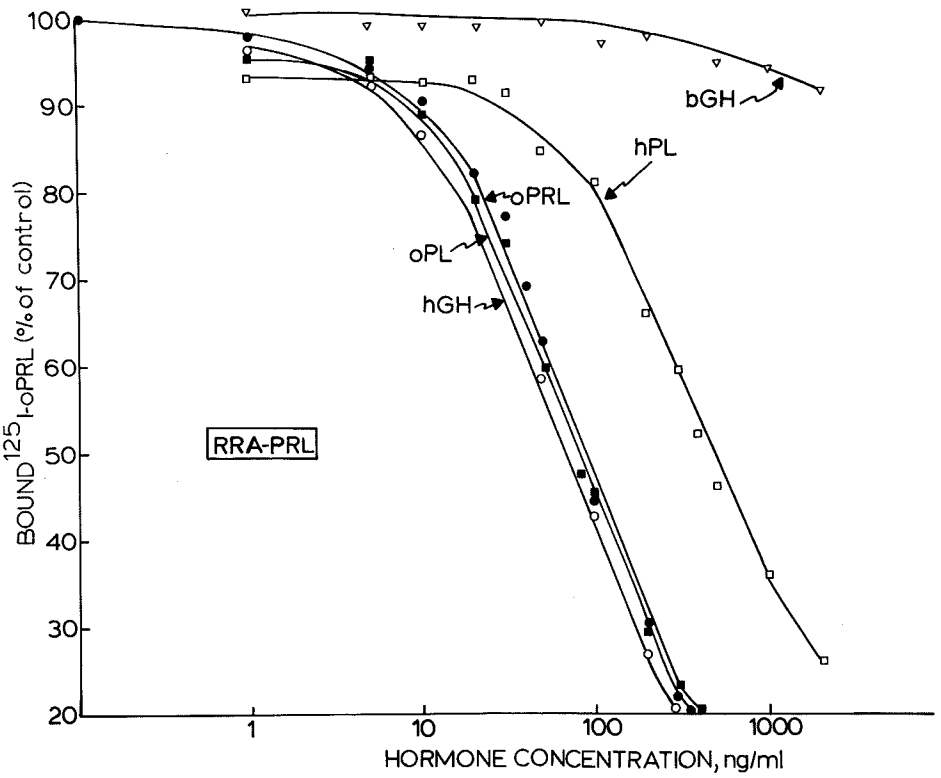

Figure 18a. Displacement curve for a highly purified preparation of oPL, hGH, hPL, oPRL and bGH in the radioreceptorassay for prolactin (RRA-PRL) using rabbit mammary gland. Rabbit mammary receptors were incubated with ^{125}I-oPRL in the presence of increasing concentration of "cold" hormone. The ordinate 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.

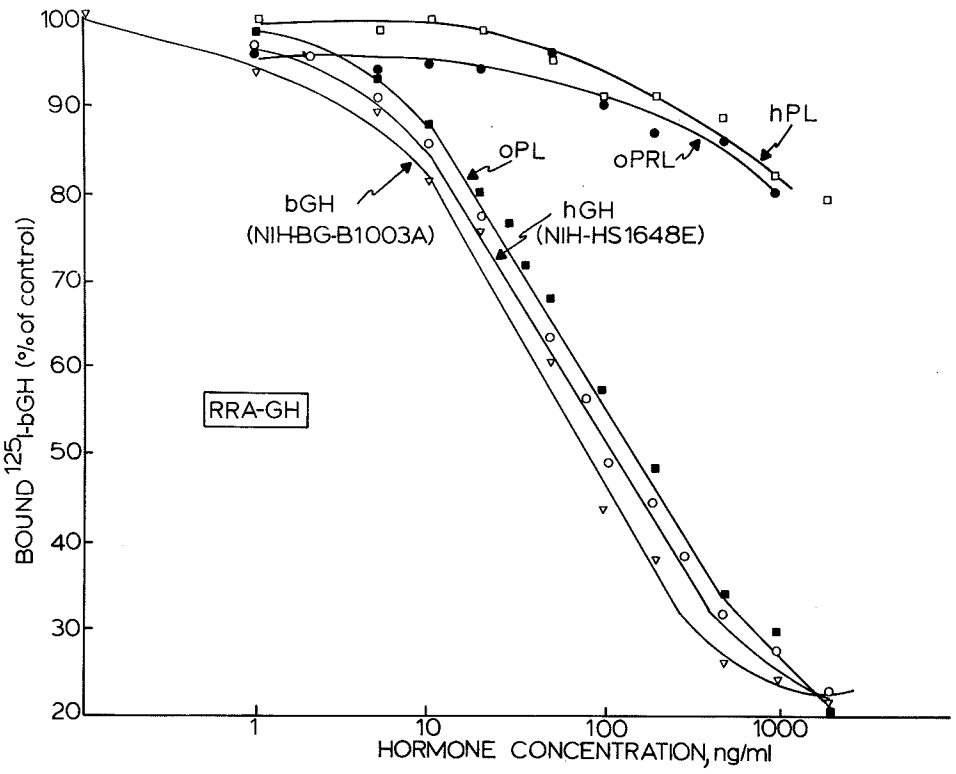

Figure 18b. Displacement curve for a highly purified preparation of oPL, hGH, hPL, oPRL and bGH in the radioreceptorassay for growth hormone (RRA-GH) using rabbit liver. Rabbit liver membranes were incubated with ^{125}I-bGH in the presence of increasing concentrations of bGH, oPL, hGH, oPRL and hPL. The ordinate represents the displacement of ^{125}I-bGH bound to growth hormone binding sites in the absence and presence of bGH, oPL, hGH, oPRL and hPL. The amount of ^{125}I-bGH bound in the absence of "cold" hormone is taken to be 100%. The abscissa represents the concentration of "cold" hormone added.

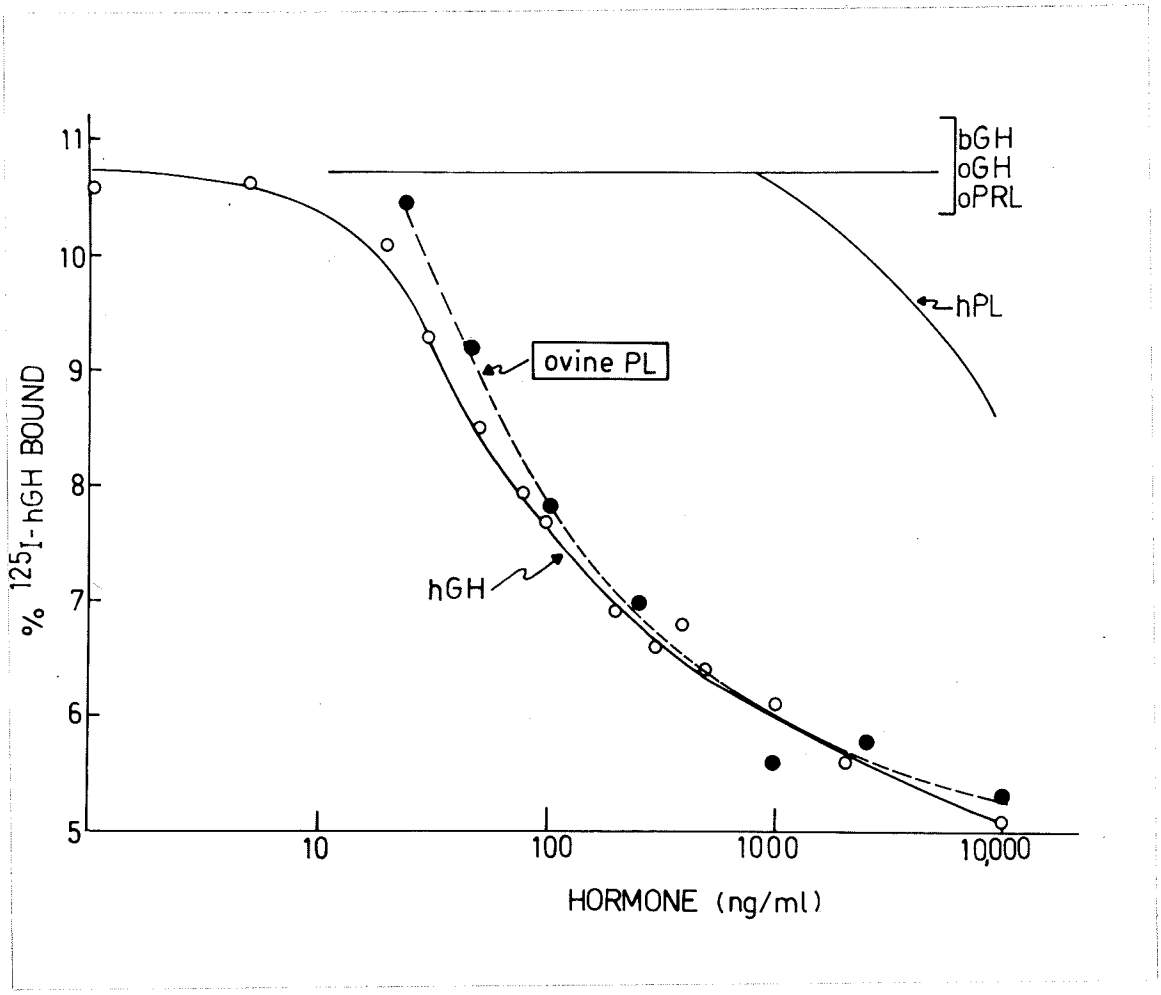

Figure 19. Radioreceptorassay for growth hormone (RRA-GH) using human liver. The specificity of the assay is shown. The ordinate indicates the % of ^{125}I-hGH bound to the human liver growth hormone binding sites in the absence or presence of "cold" hormone added to the assay tube.

SECTION VI: DISCUSSION

The main reasons for the previous failure to purify ovine placental lactogen are two fold: namely, the lack of immunological reaction of ovine placental lactogen (oPL) with antiserum to hPL, mPL, or other pituitary hormone preparations; and secondly, the absence of a simple, inexpensive, and specific assay for monitoring ovine placental lactogen. With the development of radioreceptorassays for prolactin (RRA-PRL) by Shiu et al(1973) and for growth hormone (RRA-GH) by Tsushima and Friesen (1973), which are relatively simple, inexpensive, and specific for prolactin-like and growth hormone-like respectively, it became possible to detect and quantitate oPL activity in the circulation of the pregnant ewe and also in placental extracts. By using these 2 RRA's as assay tools, we and others (Fellows et al,1974) have been able to purify ovine placental lactogen from sheep placental cotyledons.

It appears that placental cotyledons of 74 days gestation or more contain more oPL than placental tissues which are obtained after parturition. The placental lactogen content remains stable when placentas are stored at -20 C, but after one year we found that oPL content progressively decreased. Thus, frozen placental tissue of 74 days gestation or more which is stored for less than one year provides the best tissue source for oPL purification. Since the collection

of placental tissues of 74 days gestation or more is usually limited in quantity, placental tissues obtained after parturition provides a promising alternative source of oPL.

A) Extraction and Purification

In our initial attempts to purify oPL from frozen placental cotyledons, we encountered a number of difficulties which will be discussed:

1. Preliminary extraction- One major problem which was encountered with the initial alkaline extract was that it 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 as compared with extracts obtained 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, mucopolysaccharides, are suspected. When this thick extract was directly chromatographed on Sephadex G-100, resolution was poor unless small batches of placental cotyledons were used.

2. Ammonium sulfate precipitation- Fractional precipitation with ammonium sulfate (40-75% saturation) proved to be a valuable step in the purification of oPL. However, one problem associated with this procedure is the solubility rate of ammonium sulfate salt when added to the extract. Ammonium sulfate dissolves very slowly in extracts, causing a relative-

ly large percentage of oPL (25-40%) to precipitate in 40% saturated ammonium sulfate.

3. Anion exchange chromatography (Whatman DE-32)- At this stage, 10 - 20 g of protein was applied to a large DEAE-cellulose column (40 X 60 cm). For unknown reasons, 40-60% of oPL was lost in this step presumably because of irreversible adsorption in the column.

4. Cation exchange chromatography (Whatman CM-23)- One major problem associated with CM-cellulose column chromatography is the amount of oPL recovered. In our experience, the percentage of oPL loss in this procedure varied between 40-60%. The nature of this loss is not clear.

B) Analysis and Characterization

1. Analytical polyacrylamide gel electrophoresis- In alkaline polyacrylamide gel electrophoresis (pH 8.8-9.0), three stained bands were seen which were not well separated. The distance between each band being approximately 2 segments is 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 2 RRA's, some overlap in activity of the middle band was noted. Fortunately, in acidic polyacrylamide gel electrophoresis (pH 4.3-4.5), the three stained bands were more clearly separated by at least 3-4 segments. The presence of oPL was detected in eluants from segments which corresoponded to the middle

stained band. Thus, the data from acid gel support the view that the middle band in the alkaline gel corresponds to oPL.

Another question raised by the polyacrylamide gel electrophoresis is the relationship of the intensity of the stained band and the amount of protein. In practice, we assume that the intensity of the stained band is proportional to the amount of protein present and is similar for all proteins, but whether this is always the case for every protein is questionable. Therefore, the exact purity of our highly purified oPL preparation is unknown. But if we assume that the 3 bands stain equally one might estimate that the oPL preparation is approximately 30% pure.

2. Analytical gel isoelectric focusing- One of the problems associated with this sensitive technique is the determination of the time which is required to set up a steady-state gradient. In our experience, the exact time is very difficult to determine. In the present studies, we employed cytochrome C as an indicator and assumed that a steady state gradient was set up when cytochrome C passed over the anode. Whether this was the time that a steady-state was set up or not, remains uncertain.

3. Estimation of molecular weight by gel filtration- The reliability of the gel filtration technique as a means of estimating molecular weight in many instances is questionable. The presence of aromatic amino acid residues in proteins can result in the retardation of proteins on the gel filtra-

tion column, resulting in an erroneous estimate of the true molecular weight.

4. Immunological relationships. In our radioimmunoassay for oPL, the only samples which cross-reacted with oPL antisera were serum from pregnant sheep and sheep placental extracts, whereas pituitary GH and PRL, exhibited no cross-reaction. However, Handwerger et al (1974) demonstrated their oPL antisera cross-reacted with ovine growth hormone (oGH) on Ouchterlony plates. Thus, a question is raised whether Handwerger's oPL preparation is the same as ours . Although our oPL preparation did not cross-react with ovine pituitary GH and PRL in a radioimmunoassay system, it is possible that some antisera to oPL may cross-react with oGH or oPRL. Thus, further studies will be necessary to define the immunological relationship between oPL and oGH, oPRL, and other hormone preparations.

5. Bioassay of growth promoting activity. Bioassay of growth promoting activity using body weight gain in hypophysectomized rats is adequate. However, it is not completely specific, since the factors contributing to the weight gain have not been defined. Therefore, in order to establish the specific growth promoting property of oPL, other bioassays for growth hormone, such as tibial assay (Geschwind and Li,1955) will be performed.

6. Bioassay of lactogenic activity. Although the mammotropic assay using organ co-culture of rabbit mammary explants

provides evidence that oPL is lactogenic, in order to assess the proliferative action of oPL, the local pigeon crop-sac assay would be preferred. Preliminary results obtained from Dr. C. Nicoll, University of California, Berkely, demonstrated that the highly purified preparation of oPL in the pigeon crop-sac assay exhibited minimal activity(approximately 10%), was non-parallel to oPRL standard(NIH-P-S-10,26 IU/mg) used. Lactogenic activity also has been assessed by Handwerger's group. They demonstrated that oPL not only stimulated lactation in vivo in the rabbit intraductal assay but also stimulated casein synthesis in vitro in mouse mammary gland explants.

7. Displacement curve of oPL in the radioreceptorassay for growth hormone (RRA-GH) using rabbit tissue. In this assay, we employed ^{125}I-bGH and bGH as tracer and standard respectively. The reason that we did this is that bGH exhibits only somatotropic and no lactogenic activity, whereas hGH produces both activities in the receptor assays (Shiu et al,1973 and Tsushima & Friesen,1973). Therefore, using ^{125}I-bGH and bGH as tracer and standard respectively, we more confidently predicted that oPL has growth hormone activity.

8. Radioreceptorassay for growth hormone (RRA-GH) using human liver. It is especially interesting that in the radioreceptorassays for prolactin and for growth hormone(RRA-PRL and RRA-GH) using rabbit tissues, only hGH and oPL have the property of cross-reacting in both assay systems. Moreover

oPL binds to human liver receptors as well as hGH whereas hPL binds only 1/100 as well. On the basis of these observations, we think it is reasonable to suggest that ovine placental lactogen because it binds so effectively to human receptors, is likely to promote the somatotropic effects triggered by the binding of growth hormone to its receptors. It has already been demonstrated that in the human lymphocyte, non-primate growth hormones do not bind to this target tissue (Roth,1973), suggesting that the species specificity of growth hormones is evident at the level of the tissue receptors. If this is the case, then it is quite possible that sheep placental lactogen would prove to be an effective therapeutic agent in promoting growth not only in non-primates which already has been demonstrated in hypophysectomized rats but possibly also in primates as well. If this were to be the case, this would represent a finding which has important therapeutic applications.

In conclusion, during our purification and analytical steps, we failed to separate prolactin and growth hormone activity, in the oPL preparation suggesting this hormone has two intrinsic biological effects. Evidence from electrophoretic and immunological studies make it clear that the protein hormone we have isolated is not ovine pituitary prolactin or growth hormone. Additional evidence that ovine placental lactogen is distinctive is that oPL is active in both

radioreceptorassays (RRA-PRL and RRA-GH), whereas ovine pituitary prolactin and growth hormone each is active in only one radioreceptorassay: oPRL in RRA-PRL and oGH in RRA-GH. Furthermore, oPL binds to human liver receptors for growth hormone whereas other non-primate growth hormones do not. This fact suggests that oPL may have important clinical implications in the treatment of human growth hormone-deficient patients.

Comparative data on placental lactogens

Only three placental lactogens, namely hPL, mPL, and oPL have been characterized to some degree. Therefore, in the following discussion, I have summarized some data relating to these 3 hormones.

1. Chemistry

TABLE III

	hPL	mPL-1	mPL-2	oPL
Mol. wt.[1]	21,000*	21,000**	22,000**	20,000-22,000
A.A. Comp.[2]	190*	183-185**	185**	
R_f(pH 8.8-9.0)[3]	0.72**	0.52**	0.52**	0.18
PI[4]				8.8

Mol. wt.[1] - molecular weight

A.A. Comp.[2] - amino acid composition

R_f[3] - electrophoretic mobility

pI[4] - Isoelectric point

*- from Sherwood et al,1971

**- from Friesen et al,1971

The molecular weight of placental lactogen from different species (Kelly et al,1974b), rat, goat, sheep, human, monkey all are similar (20,000 - 22,000 M.W.) as

judged by gel filtration studies, whereas the molecular weight of bovine placental lactogen appears to be 45,000 M.W. and guinea pig is 60,000 M.W..

2. Secretion

TABLE IV

	hPL	mPL	oPL
1. Earliest period detected (day)			
(a) Maternal blood	30	40	60**
(b) Plac. Tissue	18*	?	31***[1]
2. Maternal conc. at term	3-10ug/ml	3-7ug/ml	0.5-1.5ug/ml**
3. Foetal conc. at term	<100ng/ml	<100ng/ml	?
4. $T_{1/2}$ phase 1	12 min.	20 min.	15-20 min.**
phase 2	75 min.	36 hours	?
5. Production rate/day	1 g	0.3 g	0.95 g***[2]
6. Placental content (mg/g wet weight)	0.3	0.07	0.1***
Average plac. wt.	500 g	150 g	400 g***
7. Plac. tissue pool turnover/day	6	20	4***[3]

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

* - Beck,1970

**-Kelly et al,1974a

***- my own studies

***[1]- The earliest placental tissue that I have studied is the 25 days of gestation (maternal caruncles or maternal attachment sites). No oPL activity detected in this early stage of pregnancy. When foetal placental tissue of 31 days of gestation was examined, oPL activity was detected but in the maternal tissue of same animal, very minimal amounts of oPL were detected. The oPL content in maternal tissues is about 1/3 that of foetal tissues. Thus, oPL is probably secreted into the maternal secretion by the foetal placental cotyledons after the attachment of the embryo to the uterus at day 30.

***[2]- The production rate per day of oPL is calculated from the first $t_{1/2}$ of 15 min. assuming an extracellular volume of 19 l..

***[4] - The placental tissue pool turnover/day of sheep is calculated on the basis that the placental content of oPL is 0.1 ug/g wet weight, production rate/day is 0.15 g. and the placental tissue weight of 400 g.

3. Biological effects

TABLE V

	hPL	mPL	oPL
1. Lactogenic effect*			
(a) Pigeon crop-sac assay	10%[1]		10%[9]
(b) Rabbit mammary intraductal assay	50-100%[2]		positive[10]
(c) Casein synthesis	50-100%[2]		90-100%[11]
2. Somatotropic effect**			
(a) Body weight assay	less 3%[3]		1.3 U/mg***[11]
(b) Tibial assay	13%[1]	positive[7]	
(c) Uptake of radioactive sulfate by rib cartilage	positive[4]		
3. Luteotropic effect (Vaginal mucification luteotropic assay)	positive[5]	positive[8]	
4. Diabetogenic effect (Lipolytic effect)	positive[6]		

* - using ovine prolactin as standard

** - using hGH as standard

*** - using bGH as standard

1 - Li, 1972

2 - Turkington, 1971; Kleinberg and Frankz, 1971

3 - Josimovich and MacLaren, 1962; Friesen, 1965

4 - Kaplan et al, 1964; Breuer, 1969; Murakawa, 1968

5 - Josimovich and Astwood, 1964; Kovacic, 1966; Henzl and Serge, 1970

[6]- Friesen,1965; Turtle et al,1966; Riggi,1966;Genazzani et al,1969

[7]- Shome and Friesen,1971

[8]- Josimovich et al, 1970

[9]- Private communication with Dr. C. Nicoll, University of California, Berkeley, U.S.A.

[10]-Handwerger et al,1974

[11]-Our own results

It appears that oPL has more prolactin-like activity in rabbit mammary assay and in the body weight gain assay than hPL and mPL. Whether oPL also has the luteotropic and diabetogenic effects remains to be demonstrated.

4. Immunological relationship

TABLE VI

	hPL	mPL	oPL
1.Antiserum to hPL	+*	+*	-***
2.Antiserum to mPL	+**	+**	-***
3.Antiserum to oPL	+***	-***	+***

* - From Josimovich & Brande,1964; Kaplan & Grumbach,1964; Friesen,1965a; Grant et al,1970.

** from Shome and Friesen,1971; Vinik et al,1973

*** from my own studies

It appears that oPL has different antigenic sites than hPL and mPL which is one of the reasons accounting for the past failure to detect ovine placental lactogen.Whether antisera to oPL raised in animals other than rabbits behave the same way remains to be demonstrated.

5. Secretion pattern

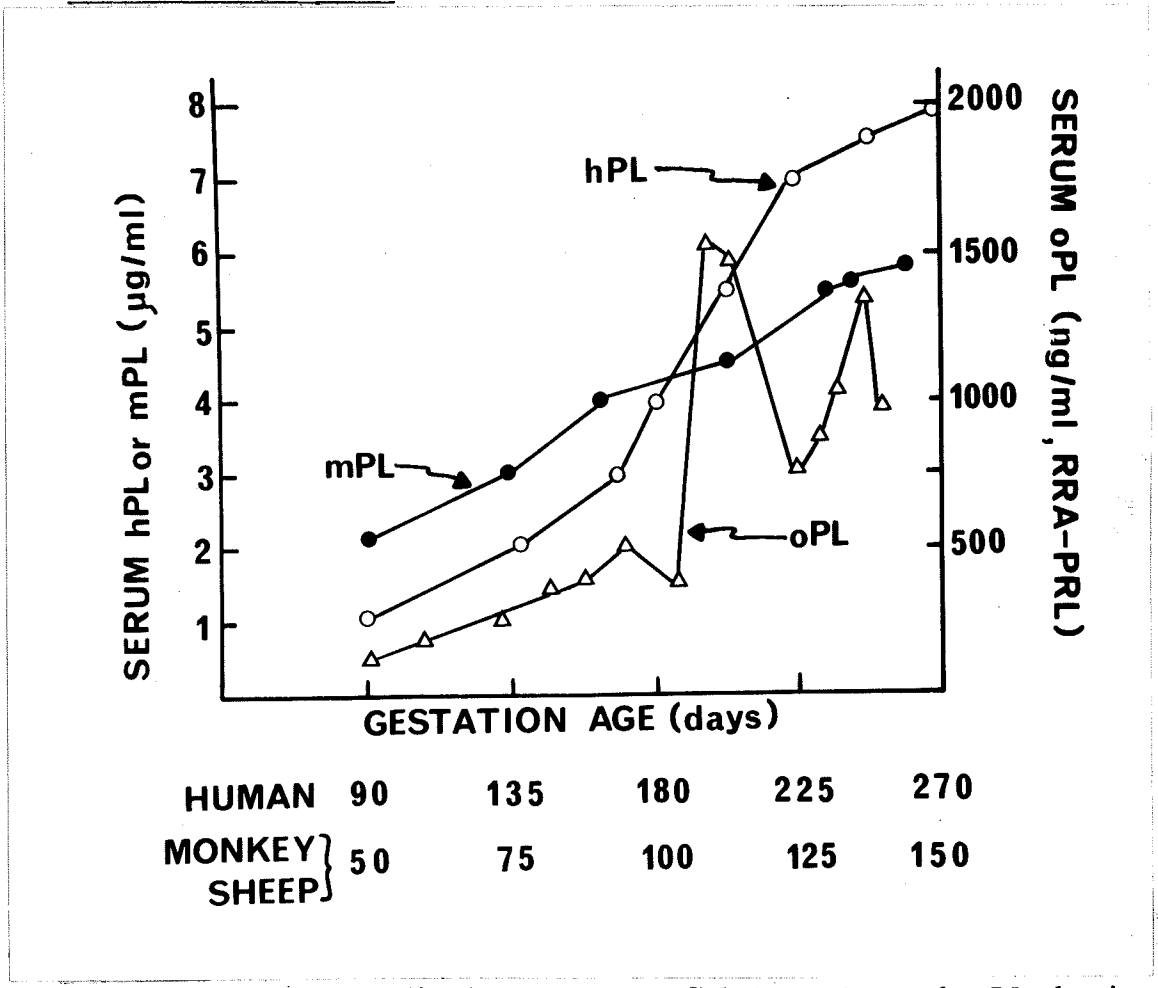

Figure 20. The secretion patterns of hPL, mPL and oPL during pregnancy. The concentrations of hPL and mPL were measured by radioimmunoassays(Friesen et al,1971),oPL by RRA-PRL (Kelly et al,1974a)

It is interesting that oPL secretion is different from hPL and mPL in that two peaks of activity are present during pregnancy. Whether each peak of activity plays a different physiological role in sheep remains unknown. Studies by Kelly et al (1974b) showed that the secreted growth hormone activity measured by RRA-GH is also very similar to prolactin activity measured by RRA-PRL. However, the ratio of PRL/GH is 3:1 or 5:1, whereas the ratio of PRL/GH in the extract and the highly purified oPL preparation is about 1:1 measured by the 2 RRA's. The reasons for the high ratio of PRL/GH in serum samples remain unclear.

It is also interesting that the concentrations of prolactin and growth hormone-like activity of serum samples of pregnant monkeys assayed by the 2 RRA's are as high as 50 ug/ml (ratio of PRL/GH is 1:1) at term. Whereas by radioimmunoassay for mPL, the concentrations reach levels of only 3-7ug/ml. Thus, whether the activities measured by the 2 RRA's are different from mPL measured by RIA also remain unclear. Furthermore, when monkey placentas were extracted and the extract measured by the 2 RRA's, a significantly higher GH and PRL-like activities were detected, at a concentration of 100 ug/g of wet weight, whereas by RIA for mPL, the content is only 70 ug/g of wet tissue. However, no significant differences in concentrations of hPL were measured in either the serum or placental extracts.

In conclusion, oPL is immunologically and chemically different from hPL and mPL, except that the molecular weight is similar with each other. Biologically, oPL is more potent than hPL and mPL in both lactogenic and somatotropic effects. Furthermore, the secretion pattern of oPL is also different from that of hPL and mPL. Whether the amino acid composition and the amino acid sequence of oPL, hPL, and mPL are also different or similar remains unclear.

Possible role of oPL in the sheep

The role of ovine placental lactogen in sheep at present is unknown. Therefore, in the following discussion, we are simply speculating about any possible effects.

1) Mammotropic effect-

Since oPL has a potent prolactin-like effect as demonstrated in the rabbit, it is possible that oPL may stimulate mammary gland development in sheep during pregnancy in preparation for lactation. Evidence to suggest that oPL might be involved in mammary development in the sheep is derived from the data obtained from experiments by Denamur and Martinet (1961). Hypophysectomy of pregnant sheep 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 controls, nevertheless some development did occur and a transient lactation took place. From these

experiments, it is suggested that during pregnancy, the placenta is able to secrete a prolactin-like substance (oPL ?) which stimulates mammary development in preparation for lactation.

2) Somatotropic (metabolic) effect-

It is not known whether ovine placental lactogen has any similar metabolic effects in the sheep as hPL does in women. Nevertheless, it appears that in ruminants, as in women, glucose utilization by maternal tissue is reduced in pregnancy (Lindsay,1971), glucose is the major energy source for the foetus (Leat,1971), and that foetal sheep plasma glucose level and glucose uptake bear a linear relationship with maternal arterial glucose concentration (Battaglia and Meschia,1972). Therefore, the role of oPL during gestation can best be considered in terms of a placentomaternal unit in which a polypeptide hormone secreted by foetal tissues (our most recent studies indicate that in fact oPL is secreted by the foetal placental cotyledons) exerts its major metabolic effect on the pregnant ewe to ensure the nutritional demand of the foetus.

Evidence supporting the above hypothesis is obtained from the fact that sheep pituitary growth hormone (maternal) secretion during the late gestational period is not elevated (Basset, Thorburn and Wallace,1970), maternal plasma glucose level is slightly depressed (Reid and Hink,1962 a and b), and the substrate requirements by the conceptus progressively

increase, thus leading to the notion that oPL may act as the "growth hormone" of pregnancy. Whether the growth hormone-like effect of oPL (as demonstrated in lower animals) would lead to an impaired glucose uptake directly in the pregnant ewe or stimulate free fatty acid (FFA) release with a resultant decrease of effective insulin remains unknown. If, oPL stimulates FFA release and exerts a contra-insulin action, then the increased ketones induced by the metabolism of FFA in the mother provide an important energy source for the foetus. As a consequence of decreased effective insulin, increased muscle proteolysis and ketone formation may be enhanced, since it is well documented that during pregnancy in the sheep muscle proteolysis and ketone formation are enhanced by restricted feeding (Beaton,1961). Thus, decreased glucose utilization and enhanced maternal gluconeogenesis induced by oPL would ensure a steady supply of glucose for the foetus.

In short, we propose that oPL might act as the "growth hormone" of pregnancy in the sheep. OPL might induce a constant tonic effect on maternal metabolism by mobilizing FFA release from adipose tissues for gluconeogenesis in the maternal liver which ensures for the foetus 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 effective insulin and induces primary catabolic effects and gluconeogenesis of oPL to ensure an adequate supply of metabolic nutrients for the foetus.

3) Luteotropic effect-

Evidence to suggest that the embryo secretes a luteotropin, possibly oPL, is derived from data obtained from the following experiments done by several investigators.

When hypophysectomy was performed in the pregnant sheep on day 2 or day 10 gestation, the corpus luteum disappeared by day 20 and the embryo was absorbed (Denamur, Martinet and Short,1966; Denamur, Torres, Kann and Short, 1972).Furthermore, when hypophysectomy was performed on day 10 of pregnancy and the embryo maintained by daily injections of progesterone (Foote,Gooch,Pope and Casida,1957; Moore and Rowson,1959; Bindon,1971), the corpus luteum still regressed completely by day 20. Thus, pituitary hormones appeared to be essential for converting the corpus luteum of the cycle into one of pregnancy. Although we cannot rule out the possibility that the conceptus may begin to secrete some luteotroic substance (oPL?), at this stage its contribution is likely to be negligible.

When an embryo is introduced into the uterus on day 12-13 of the estrus cycle, the corpus luteum is transformed into a corpus luteum of pregnancy, and the life-span of the corpus luteum is extended 5-10 days longer, whereas when the embryo is removed before day 12 of gestation, corpus

luteum regressed more rapidly within 2-3 days (Moore & Rowson, 1966 a,b,c,d). When hypophysectomy was carried out on day 30 of gestation and after the embryo had been implanted (Amoroso, 1952;Bjorkman,1965;Davies and Wimsatt,1966;Boshier,1969), the corpus luteum regressed more slowly, although the conceptus eventually aborted. However, when hypophysectomy was performed on day 60 of gestation, the weight of the corpus luteum examined 12 days later, remained at 75% of the control value, and abortion did not occur. There was no evidence that the secretory activity of the corpus luteum increased during the first fifty days of pregnancy (Edgar and Ronaldson,1958; Short and Moore,1959; Basset, Oxborrow, Smith and Thorburn,1969;Flylling 1970), but the corpus luteum is necessary for the maintenance of pregnancy during the first fifty days of gestation (Denamur and Martinet,1955). Thus, these findings indicate that the embryo is essential for the maintenance of the corpus luteum of pregnancy after day 12, but the nature of the luteotropic stimulus secreted by the embryo is unknown. The function of the embryo is to extend the life span of corpus luteum rather than to stimulate the secretory activity of the gland.

It is interesting that the time at which the conceptus acquires its luteotropic property coincides with the time when progesterone levels begin to increase (Harrison and Heap,1968; Basset et al,1968). One convincing piece of evidence suggesting that the luteotropic hormone may be oPL derives from the fact that the increase in serum progesterone

and oPL during pregnancy parallel each other (Kelly et al, 1974). However, whether oPL actually stimulates progesterone secretion requires direct experimental evidence.

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

Possible experiments to demonstrate the biological effects of oPL in sheep

1)Mammotropic effect-

The mammotropic effect of oPL may be demonstrated by local adminstration of oPL to pseudopregnant sheep. The methodology will be similar to that described by Bradley and Clarke (1956). One might also examine the effect of oPL on casein synthesis by sheep mammary explants according to the method described by Juergens et al (1965). Furthermore, if the availability of oPL permitted, oPL may be administered into hypophysectomized non-pregnant ewes to observe the effects on mammary development.

2)Metabolic effect-

The metabolic effect of oPL may be demonstrated using *in vitro* 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 sheep tissues are used instead of those from rat or rabbit.

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

3) Luteotropic effect-

The luteotropic effect of oPL in the sheep may be demonstrated by several means. First, binding studies of ^{125}I-oPL on the sheep corpus luteum could provide evidence to indicate that oPL has an effect on the corpus luteum. Secondly, by measuring progesterone levels of plasma in the ovarian vein after infusion of oPL into the ovarian artery of the hysterectomized sheep , the direct relationship of oPL and progesterone levels could be assessed. And finally, by chronic infusion of oPL into hypophysectomized, and hysterectomized pregnant sheep and measuring the relationship among the levels of progesterone, oPL, and the maintenance of the pregnant corpus luteum, we could assess the significance of oPL in maintaining the corpus luteum.

SECTION:VII SUMMARY

In summary, we have been successful in partially purifying ovine placental lactogen (oPL) and have demonstrated that it has potent growth promoting and mammotropic effects in lower animals. OPL appears to be structurally related to human growth hormone because it binds effectively to lactogenic as well as somatotropic binding sites in target tissue in animals and even more importantly in human tissues. Hence, its steric conformation must be very closely related to that of hGH. As a result it is possible that oPL may have growth promoting activity in humans.

SECTION VIII : BIBLIOGRAPHY

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