THE UNIVERSITY OF MANITOBA

PURIFICATION AND CHARACTERIZATION OF BOVINE PLACENTAL LACTOGEN

by

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'Every man in his time plays many parts'.

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LIST OF ABBREVIATIONS

PEPTIDE HORMONES

CG	Chorionic Gonadotropin
GH	Growth Hormone
LH	Luteinizing Hormone
PRL	Prolactin
PL	Placental Lactogen

PREFIX TO HORMONES

b	bovine
h	human
o	ovine
r	rat
c	caprine
m	monkey

UNITS OF MEASURE

°C	Degree Centigrade
cpm	Counts per minute
g	Gram
mg	Milligram
µg	Microgram
M	Molar
mM	Millimolar
µl	Microlitre
ng	Nanogram
l	Litre
xg	x Gravitational Force
IU	International Unit

ASSAYS AND ACTIVITIES

RRA	Radioreceptor Assay
RIA	Radioimmunoassay
% Sp.Bi.	$\frac{\text{cpm specifically bound}}{\text{total cpm in assay}} \times 100$
specific binding	bound cpm that are displaceable by excess homologous hormone

REAGENTS

^{125}I-	denotes radioactive iodine-labelled molecule
PBS	0.01M phosphate buffer with 0.9% (w/v) saline, pH 7.4
NRS	Normal Rabbit Serum
NGPS	Normal Guinea Pig Serum
SDS	Sodium Dodecyl Sulfate
PAG	Polyacrylamide Gel
IEF	Isoelectric Focusing

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ABSTRACT

Bovine placental lactogen (bPL) has been purified to homogeneity from bovine fetal placental tissue employing conventional protein purification techniques and a radioreceptor assay for human growth hormone (RRA-hGH). Analysis of the purified protein has established the molecular weight of bPL to be approximately 35,000 daltons with an isoelectric point of 5.5. The radioiodinated protein is equipotent on a molar basis to hGH in binding and displacement characteristics to growth hormone receptors of rabbit liver cell membranes. The ability of bPL to modulate hGH-specific receptors on human lymphocytes provides further evidence of the similarity of bPL and hGH. Specific binding and displacement characteristics of bPL in the RRA for prolactin (RRA-oPRL) demonstrate that it is approximately 30 percent as potent as oPRL in this system. Proliferation of Nb2 node rat lymphoma cells in culture has demonstrated that bPL is lactogenically equipotent to bPRL. An homologous radioimmunoassay developed for bPL (RIA-bPL) was unsuccessfully applied to the measurement of bPL in the serum of the fetus and the pregnant cow. Amniotic fluid and milk in midpregnancy contains no bPL. Application of the RRA-hGH to whole and fractionated serum samples has failed to uncover any hormonal activity attributable to a protein of placental origin. Biosynthetic studies with midgestational fetal cotyledonary tissue demonstrates the ability of bovine placental tissue to synthesize bPL. Immunohisto-chemical localization studies visualize bPL-like reactive sites

to chorionic tissue of the bovine placenta. In the Nb2 node lymphoma cell assay, bPL was not detected in serum samples from pregnant cows but was found in fetal serum samples.

These results demonstrate the similarity of bPL and hGH in a number of assays. The molecular weight of bPL when compared to other lactogenic and/or somatotropic hormones appears somewhat greater. The studies also provide evidence that bPL is absent from, or present in very low concentrations in, the peripheral circulation of the pregnant cow.

I: INTRODUCTION

A: THE PLACENTA

Although the placenta was at one time thought to function almost exclusively in the transfer of gaseous compounds and nutrients to the fetus we know now that it possesses many other functions as well. During its nine months of life in the human, the placenta passes through the "seven ages" of man, providing a multiple organ system which simultaneously serves fetal alimentary, pulmonary, renal, hepatic, and endocrine functions at the various stages of development. The placenta is no passive filter. Yet the fact that it separates the maternal and fetal circulations anatomically and that all exchange - gaseous, soluble, or particulate, irrespective of the direction of transfer - takes place at this interface, long perpetuated the belief that the placenta functions simply as a filter barrier, albeit a specialized one. This concept has now been superseded by the recognition that the placenta plays a selective and active role in the transfer and synthesis of substances essential for fetal well-being and development. The ability of the placenta to induce molecular modification of exogenous as well as endogenous material demonstrates its distinct metabolic identity, concerned with its own needs as well as those of the fetus. Because placental size generally correlates with fetal size and number, the requirements of the fetus have usually been considered the dominant driving force for

placental growth. However, studies dealing with placental carbohydrate metabolism indicate that at least one-third of the oxygen and glucose provided by the maternal circulation for the conceptus is used to support the metabolism of the placenta itself (Newsholme and Crabtree, 1976; Sugden and Newsholme, 1975) and that the rate of glucose transfer between mother and fetus via the placenta is much greater than would be expected on physico-chemical grounds alone, indicating that its diffusion must be facilitated by special carrier systems (Newsholme, 1977). It is also known that the amino acid concentration is consistently higher in the fetal than in the maternal blood, transfer to the fetus being against a concentration gradient (Crumpler et al, 1950). In particular, the placenta continues to grow after fetectomy and will continue to produce specific hormones even in the absence of a fetus (Petropoulos, 1973). The placenta in this context may be seen to dominate the fetus, thereby determining the growth and intrauterine lifespan of the fetoplacental unit.

While the placenta of several mammalian species has been shown to produce steroid hormones, and the now familiar term "fetoplacental unit" was coined by Diczfalusy (1964) to signify the collaborative effort of two incomplete steroid synthesizing tissues (placenta and fetus) in the biosynthesis of estrogens, the remainder of this introduction will deal briefly with only placental peptide hormones.

B: ENDOCRINE FUNCTIONS OF THE PLACENTA

Based on his success in stimulating lactation if women with oral coses of "chorinine", an extract of sow placenta, Bouchacourt (1903) was the first to postulate an endocrine function for the placenta. Shortly after this, Halban (1905) published a report on the ability of the placenta to maintain pregnancy in women in spite of ovariectomy. Following the report of the presence of a luteotropin in placental extracts (Aschner, 1913) came the description by Ascheim and Zondek (1927) of a potent gonadotropin in the urine of pregnant women capable of producing follicular growth and luteinization in the immature mouse ovary. Selye et al (1933) reported the continuation of normal pregnancy and parturition in the hypophysectomized rat, demonstrating that the pituitary gland was not necessary for the maintenance of pregnancy. The initial name applied to the urinary luteotropin identified by Ascheim and Zondek (1927), was "prolan" but this first placental protein hormone to be recognized and characterized is now known as human chorionic gonadotropin (hCG). hCG is a glycoprotein with an approximate molecular weight of 36,000 to 40,000 daltons with the carbohydrate portion accounting for 33% of its weight. hCG, like hLH, hFSH, and hTSH, consists of two subunits: labelled α and β. While the α subunit is identical for the four tropic hormones, the β subunit is specific for each. Removal of the sialic acid decreases the biological activity of

hCG and shortens its half-life (Mori, 1969; Braunstein et al, 1971).

Evidence of the appearance of hCG in urine at 10 to 12 days postovulation (Lyon et al, 1953) and in blood between the 9th (Jaffe et al, 1969) and 12th day (Marshall et al, 1968; Braunstein et al, 1973) after ovulation supports the contention that hCG is a secretory product of placental trophoblast immediately upon its differentiation from other cells of the blastocyst. Indeed, appearance of this hormone in blood and urine of a woman after she conceives marks the differentiation of trophoblast from the remainder of the blastocyst.

Using peroxidase-labeled antibody to hCG, Brody (1969) and Ikonikoff and Cedard (1973) have localized hCG in the syncytiotrophoblast of both early and full term placentas. Using anti beta-subunit serum Naughton et al (1975) was able to localize hCG as a continuous layer on the surface of syncytiotrophoblast cells. This information, in conjunction with a report by Adcock et al (1973) of the ability of hCG to inhibit the immune response of lymphocytes to phytohemagglutinin, has raised speculation that hCG may play a possible role in protecting the fetal allograft from rejection. Nygren et al (1973) measured plasma hCG, progesterone, and estradiol in a group of women with threatened abortion. They found that of the three hormones, measurement of hCG provided the best method for evaluating the prognosis of the pregnancy. With 2 exceptions, all of the

46 patients who aborted had serum hCG levels of less than 10,600 mIU/ml and all of the 23 women who did not abort had levels greater than 18,600 mIU;ml. Later in pregnancy, as the fetus grows, corpus luteum function is taken over by the placenta.

A luteotropic effect of hCG early in pregnancy is suggested by Channing (1970) and Kammerman (1974) who demonstrated hCG receptor sites in the thecal and granulosa cells of the maturing follicle and in the luteal cells of the corpus luteum. Segaloff et al (1951) observed thecal lutein changes in ovaries of non-pregnant women who had received exogenous hCG over a period of two or three weeks, beginning during the luteal phase of a spontaneous cycle. These histological findings were similar to those observed in pregnancy.

Channing (1970) has also shown that hCG stimulates progesterone secretion by granulosa cells at very low concentrations. Moreover, Cedard et al (1970) have demonstrated the stimulation of placental estrogen production by hCG.

The existence of chorionic gonadotropin in other mammals has been suggested by numerous workers. Secretion of a gonadotropin by the placentas of the rat (Matthies and Lyons, 1971; Cheng, 1975), mouse (Deansely et al, 1941), cat (Sadley, 1975), dog (Smith et al, 1974), goat (Van Rensberg, 1971), and sheep (Moore and Rowson, 1966) have been reported. A CG of placental origin in the pregnant rhesus monkey, originally reported

by Ascheim and Zondek (1928) has been confirmed by numerous researchers (Ehrhardt and Ruhl, 1933; Simpson, 1955; Tullner et al, 1966). Purification of a gonadotropin in species other than the human, how ever, has been restricted to the horse (Bourrillon and Got, 1959; Gospodarowicz, 1972) where it is referred to as pregnant mare serum gonadotropin.

An early publication by Starling (1905) dealt with the mammary gland growth of virgin female rabbits in response to extracts of rabbit fetuses and suggested the existence of a mammotropin of fetal origin. Sometime later, Madruzza (1927) reported the lactational response of virgin guinea pigs to placental homografts while Ehrhart (1936) demonstrated prolactin-like activity in extracts of human placenta.

In 1961, Fukushima prepared a human placental extract which exhibited a growth hormone-like effect. Almost simultaneously other Japanese workers obtained evidence for prolactin-like activity in placental tissue (Kurosaki, 1961; Ito and Higashi, 1961; Higashi, 1961). However, it was the report of Josimovich and MacLaren (1962), describing the immuno-chemical reaction between antiserum to hGH and a substance in the placenta and retroplacental blood, which aroused widespread interest. This report was confirmed by Kaplan and Grumbach (1964) who demonstrated that this hGH-like antigen was produced by the syncytiotrophoblast and designated the material "human chorionic growth

hormone - prolactin". It is now commonly referred to as human placental lactogen (hPL). Somatotropic (Josimovich, 1965; Florini et al, 1966), as well as luteotropic and lactogenic activities (Friesen, 1965; Josimovich and Mintz, 1968) have been demonstrated for hPL. In addition, hGH and hPL show similarities in molecular weight and amino acid composition with 85% of the 191 amino acid residues in the two molecules being identical (Niall et al, 1971; Shine et al, 1977). The chemical similarity between hPL, hGH, bGH, and oPRL, suggests that these hormones may have arisen from a common ancestral structure (Niall et al, 1971; Alojetal, 1972).

Detection of serum hPL of women who conceive occurs initially about 34 to 38 days after the LH peak, that is, 23 to 26 days after detectable levels of hCG are present (Mishell et al, 1973).

The synthesis of hPL was first demonstrated in slices of first and third trimester placentas by Grumbach and Kaplan (1964). Friesen et al (1969), in studies on the synthesis and secretion of hPL, identified hPL as one of the principal radioactive proteins in the incubation medium, where it accounted for 10 to 50 percent of the trichloroacetic acid precipitable proteins.

Suwa and Friesen (1969) showed that hPL secretion comes from a stable intracellular pool which, in the term placenta, amounts to 150 mg per 500 g of tissue.

The production rate of hPL in pregnant women has been estimated to be about 1 g per day (Solomon and Friesen, 1968; Kaplan et al, 1968).

As has been reported for hCG, Sciarra et al (1963), using both direct and indirect immunofluorescence techniques, located hPL in the cytoplasm of the syncytiotrophoblast of chorionic villi. This observation has subsequently been reported by others (Grumbach and Kaplan, 1964; Currie et al, 1966; Beck et al, 1969; Ikonikoff et al, 1971).

It appears that placental perfusion is the most critical factor in hormone synthesis and secretion since its existence ensures adequate glucose and amino acids to the placenta, and the peripheral concentrations of hPL may be indicative of placental growth and weight. A positive correlation has, in fact, been found between placental growth and hPL by Samaan et al (1971). This correlation was true not only for placental growth but for placental weight as well, indicating that peripheral hPL levels may be related to a functioning placental unit. The significance of peripheral hPL concentrations in detecting changes in fetoplacenta function is still in question. Examination of data reported by Letchworth and Chard (1972) indicates that the wide range of hPL levels in normal pregnancies precluded the possibility of conclusively correlating the prediction of subsequent fetal distress or neonatal asphyxia to abnormal hPL levels in the mother. Spellacy et al (1976)

found that hPL levels were below 4 ug/ml after the thirty-sixth week of gestation in 60% of pregnancies complicated by intrauterine growth retardation. A similar report by Gobari et al (1978) suggests an association between hPL levels of less than 5 ug/ml and an increased incidence of intrauterine growth retardation. However, because of the high number of false-negative and false-positive results, measurement of hPL levels appears to be of limited clinical value in the individual patient. The authors conclude that hPL measurement may have some usefulness in screening the patient at risk for intrauterine growth retardation, but the results reported are only similar to those obtained using 24 h urine estriol determinations and inferior to reports using ultrasound imaging techniques. Spellacy et al (1978) demonstrate a significant elevation of hPL in women with twin pregnancies at both the thirtieth (7.0 vs 6.0 ug/ml) and the thirty-sixth (9.2 vs 7.4 ug/ml) weeks of pregnancy. These elevated levels of hPL are suggested to be a potentially useful aspect of a screening profile for the detection of high-risk pregnancies, for one of the groups of women with pregnancies at risk is the one with multiple fetuses. This latter report, and ones like those by Dhont et al (1976) and Daw (1977), agree with the notion that elevated circulating placental hormone lovels are associated with an increased mass of placental tissue. Similar correlations were previously noted for hCG as well (Jones et

al, 1944; Halpin, 1970). Letchworth et al (1978) in a study of 1000 women in the third trimester of pregnancy reported that only 5.9% of their study population with hPL levels greater than or equal to 4.3 ug/ml presented with stillbirths or had fetuses below the fifth centile of weight for gestation. Their recommendation was that measurement of hPL should become part of the routine screening in the third trimester of pregnancy even though 60% of patients with low levels of hPL had normal pregnancies.

Tyson et al (1974) have shown that after the ingestion of glucose, protein, or glucose-protein by pregnant women during the second half of gestation there was no significant alteration of hPL levels. Experiments with infused glucose and arginine, alone or in combination with each other, produced results suggesting that physiologic changes in plasma glucose will not affect the secretion of hPL but may suppress hPL secretion in cases of sustained hyperglycemia. Tyson et al (1971a) demonstrated that hPL levels increased by 30% with prolonged fasting in women before the thirtieth week of gestation perhaps in response to the hypoglycemia but also possibly, as is seen with hGH, as a result of the increased release of amino acids from muscle (Tyson et al, 1971b). The hPL response may also be due to the hypoinsulinemia seen in fasting. A report by Bennett et al (1976) demonstrated that, as had been seen previously for hGH by Martin and Gagliardine (1967), hPL

restored the normal pancreatic insulin response to glucose in the hypophysectomized rat. These authors indicated that the ability of hGH, hPL, plasmin-modified hGH, and oPRL to produce weight gain in the hypophysectomized rat cannot be correlated with their ability to promote insulin secretion in response to glucose. Spellacy et al (1971) demonstrated that hyperinsulinemia resulting in hypoglycemia caused a significant elevation of hPL and suggested that the level of metabolites reaching the fetoplacental unit regulates in part the concentration of hPL found in the maternal serum. The usefulness of this, they reason, might be that if fetoplacental hypoglycemia triggers in some manner the release of lipolytic factor (hPL) so that maternal fat catabolism occurs, then maternal glucose utilization is decreased and more becomes available to the fetoplacental unit.

Spellacy et al (1966, 1971a) found that the concentration of hPL was unaffected by the time of day, sex of the fetus, presence of congenital anomalies in the fetus or maternal hemoglobin concentration. They also discovered that no significant changes in the maternal levels of hPL occurred with the infusion of oxytocin and that neither prostaglandin E_2 nor F_2 , while altering the secretion of hGH (MacLeod and Lehmeyer, 1970), had any effect on hPL concentration.

hPL possesses lactogenic activity in rabbits and pigeons and luteotropic action in the rat (Josimovich

and Archer, 1977). It may also potentiate the ability of hGH to cause growth of the tibial epiphyses in hypophysectomized rats (Josimovich and Brande, 1964). The lipolytic action of hPL is thought to be important in conserving carbohydrates during pregnancy. Talamantes (1975) reported that in the pregnant woman the basal and the hPL-stimulated rate of lipolysis in adipose tissue is higher than in the non-pregnant woman. Evidence that there may be control mechanisms specific for the synthesis or release of hPL is provided by the observation that placental slices incubated in vitro rapidly lose their ability to synthesize hPL but retain their ability to synthesize hCG (Gaspard and Franchimont, 1972).

As well as in the human, primate lactogenic placental hormones have been found in and purified from the monkey (Grant et al, 1970; Shome and Friesen, 1971). Rodent PL has been purified in the rat (Robertson and Friesen, 1975) and mouse (Talamantes et al, 1980) and identified in the hamster and chinchilla (Talamantes, 1973) and guinea pig (Kelly et al, 1976). Among the Artiodactyla, PL has been purified in the sheep (Fellows et al, 1974; Martal and Djiane, 1975; Chan et al, 1976), goat (Grissom et al, 1977), and cow (Bolander and Fellows, 1976; Roy et al, 1977; Hayden and Forsyth, 1979) and reported in the pig (Gusdon et al, 1970). Rabbit PL has reportedly been purified by Bolander and Fellows (1976). Reports by Gusdon et al

(1970) and Kelly et al (1976) suggest the presence of PL-like material in the dog placenta.

Results from animal experimentation have led to suggestions that PL may play a significant role in the physiology of pregnancy in many species.

Lyons (1944) demonstrated the synergistic action of rat placental extracts and ovarian steroids in inducing mammary lobulo-alveolar growth. Fetectomy in the rat, on day 16 of pregnancy, had no significant effect on mammary weight, DNA, or RNA content on day 21 (Desjardins et al, 1968). Complete abortion, however, reduced the weight of the mammsry glands to control, non-pregnant levels by day 21. The rat placenta, therefore, appears to make a significant hormonal contribution to mammary development.

Pencharz et al (1934) were the first to demonstrate that hypophysectomy of the pregnant guinea pig, on or after day 40 of gestation, did not lead to abortion. The pregnancy in this condition went to term and the dam possessed lactationally functional mammary glands. This data suggested that the guinea pig placenta elaborated a substance or substances with luteotropic and mammotropic qualities.

Organ co-culture experiments (Forsyth, 1972) have demonstrated the production and secretion of a placental mammotropin in the goat. This caprine placental lactogen (cPL) is detectable in the maternal circulation from the 9th week of gestation until term. Purified

cPL (Grissom et al, 1977) has an amino acid composition similar to ovine placental lactogen (oPL) and is as potent as ovine prolactin when tested in the RRA for oPRL.

oPL has been shown to be a potent lactogen which stimulates lactation in vivo in the rabbit and mouse mammary gland explants and also is a potent growth promoting protein as demonstrated in hypophysectomized rats (Handwerger et al, 1974; Chan et al, 1976). The administration of oPL into pseudopregnant rats also prevents the loss of LH-receptors in the corpora lutea and the fall of pregesterone by PGF_2 .

Pregnancy in the Cow with Special Reference to Bovine Placental Lactogen

The hormones which are primarily responsible for the maternal recognition of pregnancy and maintenance of pregnancy in the cow, as well as the interrelationship of the various factors responsible for mammogenesis and lactogenesis, is far from clear. It has been shown that in the ewe, a species in which implantation occurs on about day 16 post coitum, trophoblastic tissue extracts but not PL is the anti-luteolytic factor responsible for rescuing the corpus luteum even before nidation. Implantation in the cow, however, does not occur until the third week of pregnancy. Flint et al (1979), using a lactogenic receptor assay, were able to demonstrate the presence of bPL in the bovine blastocoele as early as day 17 of gestation. As well, Wetteman and Hafs (1973) have monitored the progesterone

profile during early pregnancy in the cow. They showed that progesterone levels were maximal at about day 18, declined gradually over the next 4 or 5 days and then increased again. Since the reestablishment of progesterone secretion (and probably rescue of the corpus luteum) occurs at about the time when bPL is first detected in the blastocyst, it is conceivable that bPL is the bovine luteotropin and/or antiluteolysin responsible for reactivation or rescue of the original CL.

It is known that ovariectomy of the pregnant cow at any time during gestation will result in abortion if no hormone replacement is initiated. Hoffman and Karg (1974) demonstrated, by monitoring plasma progesterone, that the CL of pregnancy stops functioning about 30 to 40 h before parturition and confirmed that only the CL, and not the placenta, contributed significantly to plasma progesterone. It is the combination of a precipitous drop in progesterone (from over 10 ng/ml to less than 2 ng/ml), accompanied by dramatic elevations of maternal estrone, corticoids (from 4 ng/ml to 16 ng/ml), and PRL (from 30 ng/ml to over 300 ng/ml) with fairly constant gonadotropin levels, which characterizes the final hormonal changes prior to parturition. Karg and Schams (1970) presented evidence to suggest that the prepartum PRL surge was apparently not the principal factor leading to the termination of pregnancy. Williams and Ray (1980) failed to demonstrate any antigonadotropic function of bPRL in the cow.

Elevated estrogens, on their own, are not felt to be direct inducers of parturition. While pharmacological doses of corticoids given systemically to the mother had been shown to lower progesterone levels in the third trimester and lead to abortion, moderate doses decreased progesterone but would not terminate pregnancy. As well, Hoffman (1977) reported that administration of corticoids at any time during the estrous cycle of the cow was ineffective in altering plasma progesterone levels. While Hoffman et al (1974) have concluded that LH and not PRL is the luteotropic factor in the cycling cow, Anderson (1980) has shown that hypophyseal-hypothalamic stalk section of mid-pregnancy heifers did not lead to abortion. The latter report, where pituitary gonadotropin secretion is no longer under direct hypothalamic control, suggests that maintenance of carefully controlled LH levels is not necessary for the continued functioning of the CL and, therefore, encourages speculation that LH is not the only, nor perhaps principal, luteotropin in pregnancy.

The influence of PL on mammary gland function has been suggested in several species including the cow. Indeed, in studies by Bolander and Fellows (1976) it was suggested that the secretion of bPL might be a helpful marker to select high from low milk producers based on their findings of significantly higher levels of bPL in the serum of high-yielding dairy cows when compared to lower milk yielding cows. Obviously if there were a simple index of high milk producers among cows this

would offer tremendous advantage to the dairy industry.

Schams et al (1972) have demonstrated the essential lactogenic role played by PRL at the time of the prepartum period in the cow. In their study the administration of bromocriptine (CB-154) during the last three days of gestation resulted in the virtual abolition of plasma PRL and subsequent near-failure of postpartum galactopoiesis. The effects of the ergot alkaloid if administered prior to 3 days prepartum, were again dramatic with respect to decreasing plasma PRL but were minimal in altering either lactogenesis or galatopoieses. Histological studies of the bovine mammary gland by Howe et al (1975) in which lactogenesis was induced using a combination of progesterone, 17B-estradiol and dexamethasone, led the authors to conclude that steroid treatment alone resulted in less than a complete maturational and functional response by the mammary epithelium. Further, Koprowski and Tucker (1973) have shown that basal PRL of pregnant, lactating cows was lower than that of lactating cows. The results of these reports is highly suggestive that a hormone of placental origin may well contribute to the mammotropic/lactogenic process which the cow undergoes during pregnancy.

All placental lactogens purified to date have demonstrated lactogenic activity by their ability to either bind to lactogenic receptor sites or to directly stimulate lactogenesis in the mammary gland. Many of these placental lactogens, including those found in the

human (Halban, 1905), rat (Astwood and Greep, 1938) and guinea pig (Pencharz et al, 1934) have been implicated as luteotropins. Examples of animal species with demonstrated placental lactogens which apparently do not function as luteotropins, however, include the cow (Hoffman and Karg, 1974) and goat (Cowie et al, 1963). All but one of these purified placental lactogens, that one being bovine PL, have established molecular weights in the area of 20,000 daltons.

The dairy cow in our society is bred selectively, and maintained primarily, for its ability to produce milk. Thus, any new insights into the establishment and maintenance of lactation in the cow could have important economic and social consequences. The fact that maximum milk yield is accomplished only on a regular cycle of pregnancies makes the hormones of pregnancy potentially critical factors in this regard. This consideration, along with conflicting reports of biological potency, molecular weight, and circulating levels of bPL during pregnancy provided the impetus for designing proposed studies in the areas outlined on the following page.

II: OBJECTIVES OF THIS STUDY

1. To purify bovine placental lactogen (bPL) from fetal cotyledons in sufficient purity and quantity for chemical and biological studies.

2. To characterize the physico-chemical and biological properties of pure bPL.

3. To develop a sensitive and specific homologous radioimmunoassay for bPL.

4. To study the synthesis of bPL by bovine placental tissue.

5. To measure the level of bPL in the circulation of the pregnant cow and fetus.

III: MATERIALS AND METHODS

A: PURIFICATION OF BOVINE PLACENTAL LACTOGEN (bPL)

All procedures described were carried out at $4^{o}C$ unless otherwise indicated.

Starting Material

Bovine placentae, at 200-270 days gestation as judges by fetal crown-rump length measurements (Benesch and Wright, 1952), were collected at the time of slaughter from Burns Meats Ltd. abatoir in St. Boniface. Maternal caruncles and fetal cotyledons were separated immediately and the fetal tissue and attached membranes transported to the laboratory on ice. Cotyledonary tissue was scraped from the membranes using glass microscope slides. These scrapings were the starting material for subsequent extraction and purification of bPL.

Assay for Monitoring Hormonal Activity of bPL

The purification of bPL was monitored by a slightly modified version of the radioreceptor assay for growth hormone as described by Tsushima and Friesen (1973). Preparation of the late pregnant rabbit liver membrane receptors and the iodination of human growth hormone (hGH; NIH-HS 2019G, 2.2IU/mg) were performed essentially as described in the original paper. To borosilicate glass tubes (12X75 mm) was added 100 ul of the membrane receptor (100-200 ug protein), 200 ul of 0.025 M Tris-HCl buffer, pH 7.6 containing 0.1% BSA and 10 m M $MgCl_2$, 100 ul of hGH standard or unknown sample, and 100 ul of ^{125}I-(iodo) hGH (50,000-80,000 cpm). An overnight in-

cubation of the assay tubes at room temperature with constant shaking was followed by the addition of 2 ml of cold 0.025 M sodium acetate buffer, pH 5.4 containing 0.1% BSA to each tube. The membranes were then sedimented by centrifugation at 780 x g for 20 minutes at 4°C, the supernatant decanted and the membrane bound ^{125}I-(iodo) hGH in the pellet counted in a Beckman gamma 4000 counting system.

Protein Measurement

Protein concentrations of fractions collected during purification were estimated by measuring their absorbance at 280 nm and assuming that one unit of absorbance was equivalent to a protein concentration of 0.8 mg/ml. The Biorad Protein Assay based on the method of Bradford was employed for the protein determination of the final purified product.

Concentration of Protein Solutions by Ultrafiltration

All concentration procedures were carried out at 4°C in an Amicon Diaflo system using cells of appropriate size for the sample volume to be concentrated. YM-10 membrane filters were used in all concentration steps.

Hormone Preparations

Bovine prolactin (NIH-P-B5-32.2 Iu/mg), bovine growth hormone (NIH-GH-B18-0.81 Iu/mg), human growth hormone (NIH-2019G-2 Iu/mg), and ovine prolactin (NIH-PS12-35 Iu/mg) were kindly supplied by the NIAMDD of the NIH, USA.

Iodination of Bovine Placental Lactogen

Purified bPL was iodinated by a modified method of Thorell and Johansson (1971). A 50 ul aliquot of the hormone, containing between 5-7 ug of bPL, was added to 12X75 mm borosilicate test tubes containing 25 ul of a 0.5 M NaH_2PO_4 buffer, pH 4.2. This was followed by the addition of 5 ul of a 1 mg/ml solution of lactoperoxidase (Calbiochem grade B) in 0.05 M phosphate buffer, 15 ul of carrier-free $Na^{125}I$ (New England Nuclear; 0.6 m Ci), and 5 ul of a 1:15,000 dilution of 30% H_2O_2. The reaction tube was agitated and allowed to stand for 5 min. After this time an additional 10 ul of ^{125}I was added (0.4 m Ci) along with 5 ul of the dilute H_2O_2. The tube contents were again mixed and allowed to stand for 5min. before a final 5 ul of H_2O_2 was added to the reaction mixture. Following a final 5 min incubation, 1 ml of PBS buffer, pH 7.4 was added to the tube and the entire contents then layered onto a 2X50 cm column of Sephadex G-100 gel previously equilibrated with PBS buffer. Elution of the radioactive sample from the column was carried out with PBS buffer at a flow rate of 55 ml/hr. The 3 ml fractions of effluent were collected in glass tubes containing 3 drops of PBS buffer with 2.5% BSA. Eluted fractions were then scanned for radioactivity using a geiger counter.

Iodination of Bovine PRL and Bovine GH

Both bPRL (bPRL; NIH-P-B5; 32.2 IU/ng) and bGH (bGH NIH-GH-B18; 0.81 IU/ng) were iodinated by the

lactoperoxidase method as described for bPL with the exception that the phosphate buffer employed had a pH of 7.0 instead of 4.2. Purification of labelled hormone was carried out by gel exclusion chromatography as for bPL.

Specific Activity of Iodinated Hormones

Determination of the specific activity of iodinated hormones involved diluting 10 ul of the final reaction mixture with 10 ml of Tris-HCl buffer, pH 7.6 containing 10 mM $MgCl_2$. Two, 100 ul aliquots of this 1:1000 dilution were added separately to two glass test tubes, precounted in a gamma counter, and then mixed with 100 ul of a Tris-HCl buffer, pH 7.6 containing 2.5% BSA and 2 ml of a 10% trichloracetic acid (TCA) solution. After an overnight incubation at $4^{o}C$ the tubes were centrifuged at 780 x g for 20 min, the supernatant decanted, and the precipitate counted in the gamma counter. The incorporation of radioactivity into protein is expressed as the percentage of the TCA precipitable radioactivity over the precount radioactivity. The specific activity of the iodinated hormone is defined as the total precipitable count divided by the amount of protein used for iodination. The iodination of purified bPL was accompanied by a 40-60% incorporation of the radioactive iodine yielding a tracer with specific activity between 80-100 u Ci/ug protein. Specific binding of the tracer to rabbit liver membrane receptors was usually in the range of 15-20% and only

when in this range was it used for subsequent studies.

B: PHYSICO-CHEMICAL CHARACTERIZATION OF BOVINE PLACENTAL LACTOGEN

Analytical gel electrophoresis

A slightly modified version of the method for polyacrylamide gel electrophoresis described by Davis (1964) was employed. Purified bPL was applied to two tubes of 7.2% acrylamide gel. Basic Fuchsin was applied to a control gel tube, and electrophoresis of all tubes conducted at pH 4.5. One bPL gel and the Basic Fuchsin gel were then stained with 0.115% Coomassie Brilliant Blue R-250 in a solution of 25% ethanol and 8% acetic acid. Destaining of the gel was carried out in a test tube with 7% acetic acid. The remaining gel containing bPL was cut serially and the individual segments were eluted overnight in 1 ml of 0.1 M Tris-HCl, pH 7.6 containing 0.1% BSA at $4^{o}C$. The eluates were subsequently analyzed by the RRA for GH.

Analytical gel isoelectric-focusing

Analytical thin-layer polyacrylamide gel isoelectric focusing was performed using an LKB 2117 multiphor apparatus and LKB Ampholine PAG plates with a pH range of either 3.5-9.5 or 4-6.5. The gel lanes to be stained were fixed in 11.5% TCA and 3.4% sulphosalicylic acid prior to staining in 0.115% Coomassie Brilliant Blue R-250 in a solution of 25% ethanol, 8% acetic acid solution prior to drying and photography. Gel lanes to be assayed for hormone activity were cut into 0.5 cm ser-

ial slices immediately after electrophoresis and the individual slices eluted in 1 ml of 0.025 M Tris-HCl, pH 7.6 buffer containing 0.1% BSA overnight at 4°C. A blank gel lane was sliced in an identical manner at the same time, the slices eluted in 1 ml of a distilled water and the pH of the eluates determined by pH electrode.

Analytical sodium dodecyl sulfate polyacrylamide slab gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out as described by Laemmli (1970) with slight modification. A 12% acrylamide resolving gel and 3% acrylamide stacking gel were prepared in the Biorad Vertical Slab Electrophoresis Cell System. Electrophoresis was conducted at a constant current of 10 milliamperes. Sample preparation prior to application to the gel involved boiling the protein solution for 1-2 min in the presence of 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and a small amount of bromophenol blue. The electrophoresed gel was stained in 50% TCA containing 0.25% Coomassie Brilliant Blue R-250 for 1-2 hours, then destained in a solution of 30% methanol, 7 % acetic acid, and finally vacuum dried prior to photography or autoradiography.

Isoelectric Focusing in Granular Gel

The isoelectric focusing of serum samples, placental extracts, and radioactive tracers in Sephadex gel was carried out using Sephadex IEF gel (Pharmacia)

with either Pharmalyte (Pharmacia) or Ampholine (LKB) as the carrier ampholyte source. An LKB 2117-501 Ampholine Electrofocusing Kit in conjunction with an LKB 2103 power supply was employed. The experimental procedure followed the basic outline described by Wahlstrom and Bjorkman (1979) and always involved addition of the sample to the gel during the swelling process. Following the gel division with the fractionation grid, pH measurements were made directly of all lanes by means of a surface pH electrode and selected lanes were then acraped out of the grid with a spatula and eluted in individual 10 ml syringes fitted with glass wool plugs.

Tissue Extraction

Tissue extracts (except placental employed in the studies designed to characterize the antiserum to bPL were all obtained in the manner described below. The entire procedure was carried out at $4^{o}C$. Fresh tissue, obtained within 30-60 min of slaughter of the animal, was minced finely with scissors. An equal volume:weight mixture of 50 m M NH_4HCO_3 buffer, pH 8.0 and pieces of tissue (usual weight approximately 10 g) was homogenized using a Polytron PT-10 homogenizer (Brinkman Instruments, Inc. Westbury, N.Y.) at full speed for 1 min. The homogenate was subsequently centrifuged at 14,000 rpm in a Beckman JA-20 rotor for 30 min and the supernatant used for experimentation.

Blood samples

The majority of steer, cow, and fetal serum sam-

ples were harvested from blood samples obtained at a local meat packing plant at the time of the animal's slaughter. Blood collected in this manner was a mixture of venous and arterial pools. Blood samples were allowed to stand for 6 h at 4°C before being centrifuged and having the serum harvested. The time of pregnancy at which the cow was slaughtered was estimated by measuring the fetal crown-rump length and relating that dimension to gestational age as described by Benesch and Wright (1952). Other serum samples were obtained from blood collected by venipuncture at known times of gestation at the University of Guelph. All sera were stored at -20°C until assayed.

Milk samples

All milk samples were collected at the afternoon milking of cows with known breeding dates at the University of Manitoba.

Amniotic fluid samples

All amniotic fluid samples were obtained from slaughtered cows and the time of gestation, again estimated on the basis of fetal crown-rump length.

C: RADIOIMMUNOASSAY FOR BOVINE PLACENTAL LACTOGEN

Generation of Antiserum to bPL

Antibodies to bPL were raised in a young, male New Zealand white rabbit (2-3 kg) by immunization of the rabbit with purified bPL. Preparation of bPL for immunization entailed the complete suspension of the antigen solution in an equal volume of complete Freund's adjuvant

immediately prior to its injection. The first step of the process involved the intradermal injection of 1 ml of a 60 ug/ml solution of bPL into sites along the left half of the rabbit's shaved back. Two weeks later the shaved, right half of the rabbit's back was wmployed as the location for an additional 20 intradermal injections of 50 ug of bPL in 1 ml of solution. The third inoculation and subsequent booster shots involved the subcutaneous injection of 1 ml of a 50 ug/ml solution of bPL in the dorsal shoulder and neck regions of the rabbit at 2 week intervals. The rabbit was bled biweekly beginning at the third inoculation from either a marginal ear vein or artery and the blood allowed to clot at $4^{o}C$ for 4 h. The serum was then removed from the clotted specimen and used as the source of anti-bPL for subsequent studies.

Radioimmunoassay for bPL

All incubations were at $4^{o}C$ and all samples, bPL, standard, and tracer dilutions were in RIA buffer (PBS buffer, pH 7.6 with 2.5% BSA and 50 mM EDTA). The double-antibody radioimmunoassay (RIA) procedure was similar to that described by Beck et al (1967). To 12X75 mm borosilicate glass test tubes, was added 0.6 ml of RIA buffer, 0.1 ml of a 1:1000 dilution of anti-bPL antiserum in normal rabbit serum (1:450), and either 0.1 ml of sample or bPL standard. The tube contents were mixed and allowed to incubate overnight. On the following day, 0.1 ml of ^{125}I-bPL (50,000-80,000 cpm)

was added and the tubes mixed and allowed to incubate overnight again. On the third day, 0.1 ml of sheep anti-rabbit gamma globulin (1:25) was added to each tube, mixed into the contents, and incubated overnight. On the fourth day 2 ml PBS buffer was added to each assay tube, the tubes centrifuged at 780 x g for 30 min, the supernatant decanted, and finally, the pellets were counted in a gamma counter (Beckman gamma 4000).

Radioimmunoassay for bPRL

To 12X75 mm borosilicate glass tubes was added 0.6 ml of RIA buffer, 0.1 ml bPRL standard or sample, 0.1 ml of anti-bPRL antiserum (45 May 26, '71; 1:10,000 in normal rabbit serum at 1:450), and 0.1 ml of ^{125}I-bPRL (50,000-80,000 cpm). The tube contents were incubated overnight at 4°C before adding 0.1 ml of sheep anti-rabbit gamma globulin to each assay tube. A second 4°C overnight incubation at this point was followed by addition of 2 ml PBS buffer to each assay tube, centrifugation of the tubes at 780 x g for 30 min, decantation of the supernatant, and finally, counting of the pellets in a gamma counter.

Radioimmunoassay for bGH

The protocol employed here was identical to that for the RIA for bPRL except that bGH standards replaced bPRL standards, anti-oGH antiserum (1:1000 in normal rabbit serum at 1:450) replaced anti-bPRL antiserum, and ^{125}I-bGH (50,000-80,000 cpm) replaced ^{125}I-bPRL.

D: IMMUNOHISTOCHEMICAL VISUALIZATION OF BOVINE PLACENTAL LACTOGEN

Immunohistochemical Localization of anti-bPL Reactive Material in Placental Tissue

Placental tissue from a cow in her second trimester of pregnancy was obtained within 30 min of slaughter. Maternal and fetal components were separated and a 2 cm^2 piece of the cotyledon from one placentome was immediately deep frozen on dry ice. This tissue was trimmed, positioned for cryostat sectioning at -20°C, and cut into 5 u thick slices. The tissue sections were mounted on standard glass microscope slides precoated with gelatin. (All subsequent processing of sections was carried out at room temperature. PBS buffer used in any of the following procedures was always without added azide.) The mounted sections were fixed in Lillies' buffered formalin, pH 7.4 for 5 min, washed for 15 min in running tap water, placed in a solution of 50% ethanol-3% H_2O_2 for 5 min to neutralize any endogenous tissue peroxidases, and again washed in tap water for 15 min. The slides were allowed to drip dry for a few minutes before being placed in a humid container where they were flooded with normal goat serum (1:10 in PBS buffer with 2.5% BSA), covered, and allowed to sit undisturbed for 1 h at room temperature. The sections were washed for 10 min in PBS buffer, allowed to drip dry until just moist, and returned to the humid chamber where the primary antiserum was added dropwise

to flood the sections. All primary antisera (anti-bPL, -bPRL, -oGH and NRS) were diluted to a working concentration of 1:500 in PBS buffer with 2.5% BSA. Following a 24 h incubation with the first antibody, the slides were washed for 15 min in PBS buffer prior to the dropwise addition of goat antiserum to rabbit gamma globulin (Cappel Laboratories, Inc.; 1:10 in PBS buffer with 2.5% BSA). A 1 h incubation in the humid chamber in the presence of this second antibody was followed by a 10 min wash of the slides in PBS buffer. The soluble peroxidase-anti-peroxidase solution (1:100 in PBS buffer with 2.5% BSA) was applied to the sections for 1 h and the sections were subsequently washed in PBS buffer for 15 min. After being allowed to drip dry, the slides were set for 1 min in a staining jar containing 150 ml of a 60 mg% solution of 3,3'-diaminobenzidine tetrahydrochloride monohydrate (Aldrich Chemical Company, Inc.) in 50 m M Tris-HCl buffer, pH 7.4 to which 3-4 drops of H_2O_2 had just been added. This staining procedure was followed by a thorough wash of the slides in running tap water. The sections were then counterstained for 30 sec in cresyl violet, washed well in tap water, dehydrated in a series of alcohol solutions ranging in strength from 50%-100%, then cleared in two changes of xylene, and finally mounted with a coverslip and Permount.

Absorption of Primary Antiserum

The primary antiserum, at a final dilution of 1:500

in PBS buffer with 2.5% BSA, was incubated at 4°C overnight in the presence of excess absorbing hormone. Anti-bPL antiserum, when preabsorbed, was incubated with either 10 ug bPL or 50 ug of bGH or bPRL. Antisera to bPRL and oGH were preabsorbed with 10 ug bPRL or 10 ug bGH respectively in the control slides. The incubation mixture was centrifuged at 1000 x g for 30 min following the incubation and the supernatant was used to flood the placental sections under examination.

E: STUDIES ON THE BIOSYNTHESIS OF BOVINE PLACENTAL LACTOGEN BY FETAL PLACENTAL TISSUE in vitro

Placental tissue from a cow in her seventh month of pregnancy (based on fetal crown-rump length measurement) was collected within 30 min of the time of slaughter. The placenta was then separated into maternal and fetal components and about 5 g of the fetal coryledon from one placentome was rinsed in 50 ml of Dulbecco's Modified Eagle Medium (DMEM, Gibco, N.Y.) on ice for 90 min. The tissue was then blotted dry and 1.5 g minced thoroughly with scissors. The minced material was added to 5 ml of Earl's Balanced Salt Solution (EBSS; Gibco) containing 5% complete medium (where complete medium consists of Eagle medium without methionine, 10 ug/ml insulin, 10% FCS, 0.35% glucose, 1% gentamycin) and 100 u Ci/ml L-(^{35}S) methionine (1200 Ci/mmol specific activity). The incubation was carried out for 6 h at 39°C while shaking in an atmosphere of 95% O_2-5% CO_2. The suspension was centrifuged to pellet

the pieces of tissue and the supernatant saved. The pellet was washed twice with EBSS and homogenized with a polytron PT-10 homogenizer for 1 min on ice in the presence of 2 ml Tris-NaCl buffer, pH 7.4 containing 2 mM phenylmethylsulfonylfluoride (PMSF) and 1% Triton X-100. Homogenization was followed by a 30 sec sonication procedure. Both the tissue homogenate and original incubation supernatant were then centrifuged at 100,000 x g for 1 h. Supernatants were decanted and stored at -20°C until tested.

Incorporation of ^{35}S-methionine into newly synthesized protein

Ten ul aliquots of each 100,000 x g supernatant were placed onto glass fiber filter discs. The discs were taken through two changes of cold 10% TCA on ice and then rinsed in two changes of cold absolute ethanol on ice. The filters were placed into glass scintillation vials and dried overnight in a hot air oven. After drying, 5 ml of Omnifluor (NEN) was added to each vial and the ^{35}S-methionine containing protein counted in an LKB 1216 Rackbeta liquid scintillation counter.

Immunoprecipitation of ^{35}S-met bPL in placental tissue homogenate and incubation medium

Procedure conducted in 2 ml Eppendorf microfuge tubes

To 100 ul of each 100,000 x g supernatant was added 100 ul of 0.01 M Tris-HCl in 0.9% NaCl (TBS), pH 7.4 containing 2 m M PMSF and 1% Triton X-100 (TX) and either 1 ul or 5 ul of heat inactivated antiserum

or normal rabbit serum. After an overnight invubation at 4°C Pansorbin reagent was added to the tubes in either 20 ul (to the 1 ul antiserum tubes) or 100 ul (to the 5 ul antiserum tubes) aliquots. Mixing and a 15 min incubation on ice were followed by a double wash in 1 ml of TBS pH7.4/PMSF/TX buffer with 3 M KCl and a final wash in 1 ml of TBS pH 7.4/PMSF/TX buffer. A 50 ul aliquot of the SDS cocktail (2% SDS, 2% 2-mercaptoethanol, bromophenol blue) was added to the precipitate and the well-mixed suspension boiled for 1-2 min. The mixture was subsequently centrifuged at room temperature and 5 ul of the supernatant added to a scintillation vial containing 5 ml Aquasol (New England Nuclear) and counted in an LKB Rack Beta scintillation counter. An additional 20 ul aliquot of the final boiled supernatant was analyzed in the polyacrylamide slab gel system containing SDS as described previously. The gel was stained with Coomassie Brilliant Blue R-250 and, following destaining, was rinsed in 2 changes of dimethylsulfoxide (DMSO; 30 min each), followed by a 3 h contact with a DMSO solution containing 20% (w/v)(2,5-diphenyloxazole (PPO; Amersham)) and then 2 changes of water (30 min each; Laskey and Mills, 1977). The gel was finally dried under vacuum and exposed to x-ray film at -70°C.

F: BIOLOGICAL CHARACTERIZATION OF BOVINE PLACENTAL LACTOGEN

Binding of ^{125}I(iodo)-bPL to rat liver membrane lactogenic receptor sites

Late pregnant rat liver membranes were prepared in a manner similar to that employed for the preparation of rabbit liver membranes described by Tsushima and Friesen (1973). Incubation of bPL tracer with the membrane preparation (100-200 ug protein) was conducted for 18 h at 22°C in the presence of 0.025 M Tris-HCl, pH 7.6 containing 0.1% BSA and 10 mM $MgCl_2$. This was followed by centrifugation of the membrane residues at 780 x g for 20 min and then washing and centrifugation of the pellet in two separate changes of 3 ml of the incubation buffer. The membrane pellet was finally incubated for 30 min on ice with 1 ml of 5 M $MgCl_2$ to strip the bound tracer from the membranes. The suspension was then centrifuged to sediment the membranes and the decanted supernatant was dialysed overnight against distilled water. Aliquots of the dialysed supernatant were analyzed by SDS polyacrylamide gel electrophoresis (as described) and the dried gel exposed to X-ray film to produce the final autoradiogram.

<u>The evaluation of the ability of bPL to modulate hGH receptor concentrations of human IM-9 lymphocytes</u>

The protocol employed in this receptor modulation experiment is a modified version of the one reported by Lesniak et al (1973). IM-9 lymphocytes were harvested from continuous suspension culture medium (RPMI-1640; GIBCo), containing 10% FCS and penicillin and streptomycin, by centrifugation of the cultures and resuspension of the pelleted cells in a minimal amount of cul-

ture medium. Cell density was adjusted to about $35X10^6$ cells/ml. To 12X75 mm plastic tubes was added 300 ul of the cell suspension, 100 ul of 25 mM modified HEPES buffer, pH 7.0 (where modification involved the addition of 1% BSA, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM Na acetate, 10 mM dextrose, and 1 mM EDTA to the HEPES) in RPMI medium, and 100 ul of either hGH or bPL. An overnight incubation with constant agitation at 22°C was followed by the addition of 2 ml of modified HEPES buffer and a 10 min centrifugation at 780 x g. After decanting the supernatant the tubes had 200 ul of modified HEPES buffer, pH 7.0 and 100 ul of either hGH (1 ug/ml) and ^{125}I-hGH (80,000-100,000 cpm) or simply 100 ul of ^{125}I-hGH added to the pelleted cells. Cell resuspension and continued suspension at 22°C was then carried out for 2 h after which time 2 ml of cold modified HEPES buffer, pH 7.0 was added to each tube. A subsequent centrifugation of the assay tubes at 780 x g for 10 min was followed by the determination of the amount of ^{125}I-hGH bound to the pelleted cells by counting the radioactive pellet in a Beckman 4000 gamma counter.

IV: RESULTS

A: PURIFICATION OF BOVINE PLACENTAL LACTOGEN (bPL)

1) Assay Employed to Monitor the Hormonal Activity of bPL During Purification

Figure 1 shows the effect of purified bovine pituitary hormones (growth hormone and prolactin) and a crude bovine placental extract in a RRA for GH (RRA-hGH). While bPRL inhibits the binding of ^{125}I-hGH only minimally, both bGH and placental extract inhibit the binding of the tracer to membrane receptors. The assay sensitivity is about 10 ng/ml. All displacement of ^{125}I-hGH in the RRA-hGH by a component of placental origin is considered to be bPL.

2) Extraction of bPL from Cotyledonary Tissue Scraped from Fetal Membranes

Homogenization of the 1 kg of scrapings in an equal volume of 0.05 M NH_4HCO_3 buffer, pH 8.2 containing 2 mM PMSF and 5 mM benzamidine was carried out using a Polytron PT-10 homogenizer at full speed for 1-2 min. This homogenate was centrifuged at 13,000 rpm for 30 min in a JA-21 rotor and the supernatant filtered through 8 layers of cheesecloth prior to ammonium sulfate precipitation.

Ammonium Sulfate Precipitation of bPL

The three stages of ammonium sulfate precipitation began with the slow addition of fine, solid ammonium sulfate to the pH 8.2 extract to a final concentration of 35% saturation. Following 2 h of stirring

Figure 1

Radioreceptor assay for growth hormone or growth hormone-like activity. Comparison of displacement curves for bPRL (◊), bGH (□), and bovine placental extract dilutions (○) with hGH standards (△) in the somatotropic RRA using rabbit liver membranes as the receptor source and ^{125}I-hGH as tracer (RRA-hGH).

Fig.1

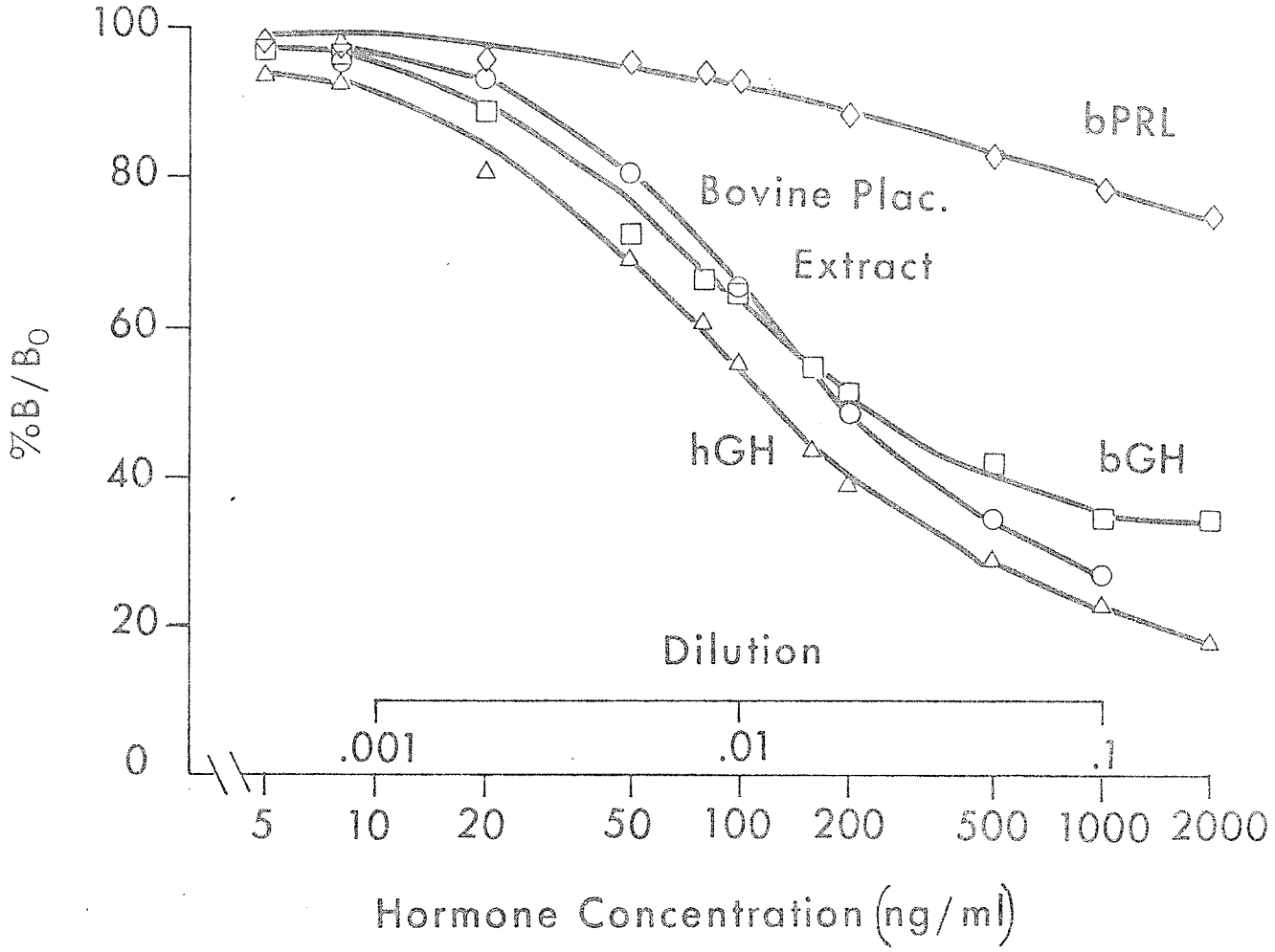

100
80
60
40
20
0
%B/B0
bPRL
Bovine Plac.
Extract
hGH
bGH
Dilution
.001
.01
.1
5
10
20
50
100
200
500
1000
2000
Hormone Concentration (ng/ml)

Figure 2

Gel filtration on Sephadex G-100 of 25 ml of the re-dissolved 45-55% ammonium sulfate precipitate. The gel column (6X70 cm) was equilibrated and eluted with 0.005 M potassium phosphate buffer, pH 6.5 at a flow rate of 40 ml/hr. Activity of the eluted fractions was determined in the RRA for hGH. Receptor active fractions from four such runs were pooled as marked and concentrated by ultramembrane filtration. The combined concentrated pool was reapplied to a freshly packed Sephadex G-100 column of the same dimensions using the same eluting conditions. The inset shows this rechromatography. Fractions as marked in the inset were pooled and applied to the hydroxylapatite column.

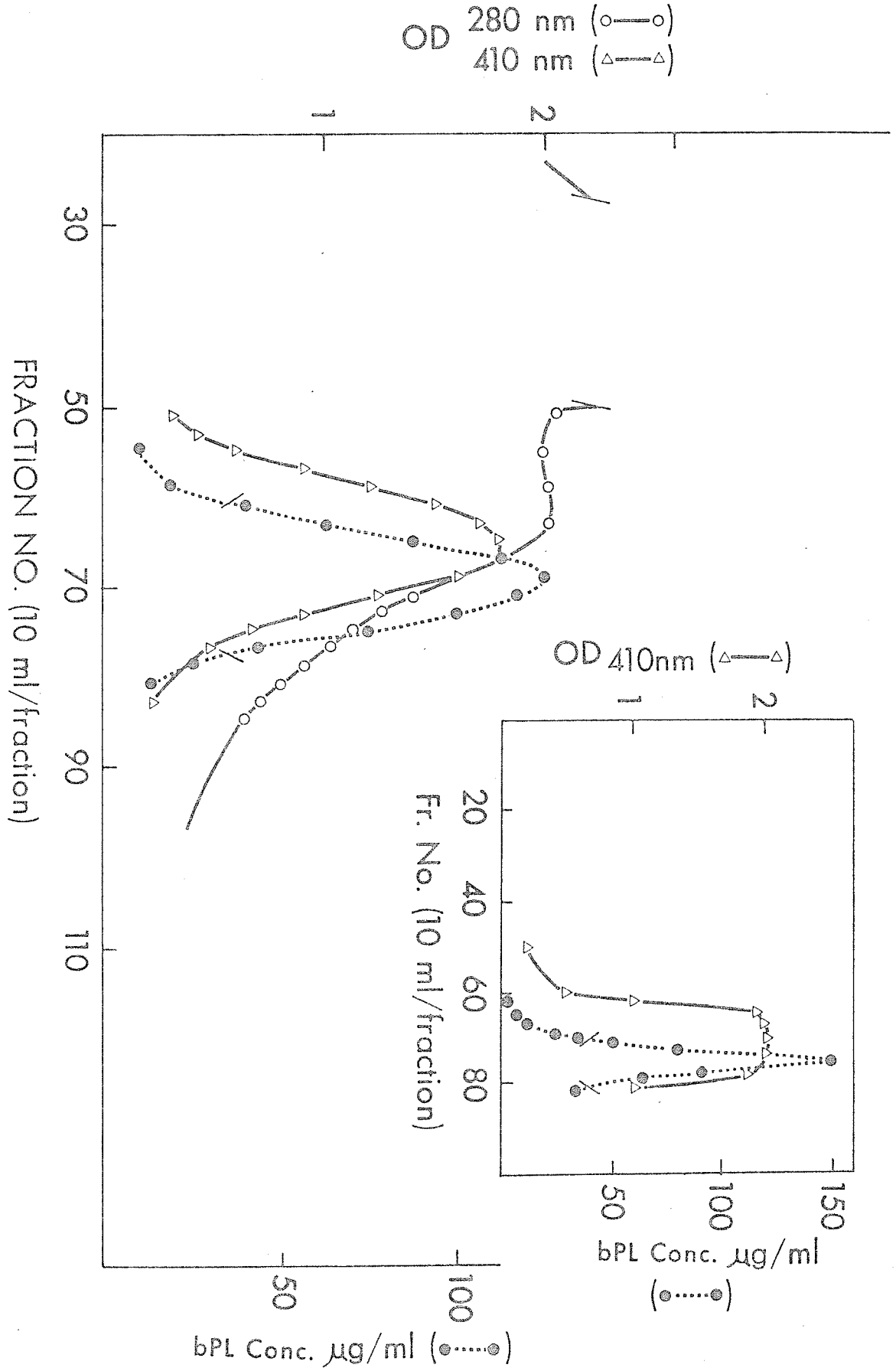

OD 280 nm (o—o)
410 nm (△—△)
1
2
30
50
70
90
110
FRACTION NO. (10 ml/fraction)
50
100
bPL Conc. μg/ml (●·····●)
OD 410nm (△—△)
1
2
20
40
60
80
Fr. No. (10 ml/fraction)
50
100
150
bPL Conc. μg/ml
(●·····●)

Fig. 2

at 4°C the fine precipitate was centrifuged in a Beckman JA-14 rotor at 13,000 rpm for 30 min and the supernatant adjusted to 45% saturation again by the slow addition of solid salt. An overnight stirring of this solution was followed by centrifugation of the mixture at 13,000 rpm for 30 min. The concentration of ammonium sulfate in the supernatant was increased to 55% with the solid salt, stirred for 8 h and then centrifuged at 13,000 rpm for 30 min. The resultant precipitate was dissolved in distilled water in preparation for application to the Sephadex G-100 gel.

Sephadex G-100 Exclusion Chromatography

The distribution of bPL after gel filtration of the 45-55% ammonium sulfate precipitate on a Sephadex G-100 column is shown in Figure 2. Most of the hormone eluted at about 2 times the void volume. These fractions from 4 columns were pooled, concentrated, and subjected to a second gel filtration on a similar column.

Hydroxylapatite (HTP) Column Chromatography

The fractions containing bPL were pooled after the second gel filtration and were loaded onto the HTP column which was previously equilibrated with 0.005 M phosphate (Figure 3). The column was washed with a stepwise increase of the phosphate buffer to 0.05 which resulted in the elution of a large amount of protein but little bPL. The latter was eluted with a linear phosphate gradient from 0.05 M to 0.2 M. The fractions containing bPL were concentrated and dialyzed

Figure 3

Hydroxylapatite chromatography of the pooled Sephadex G-100 fractions. The 6X6 cm HTP column was equilibrated with 0.005 M potassium phosphate buffer, pH 6.5 prior to application of the 105 ml G-100 pool. Sample application was followed by a column wash with 0.05 M potassium phosphate buffer, pH 6.5 and then elution of adsorbed activity with a linear potassium phosphate buffer gradient from 0.05M-0.2M. Activity of the eluted fractions was measured in the RRA for hGH. Fractions as marked were pooled, concentrated by ultramembrane filtration, and dialyzed against 0.005 M sodium phosphate buffer, pH 5.8 prior to application to the SP-Sephadex column. The linear elution gradient was begun before the protein profile reached baseline in order to remove bPL as quickly as possible from the column and also to minimize the spread of the eluted peak of bPL.

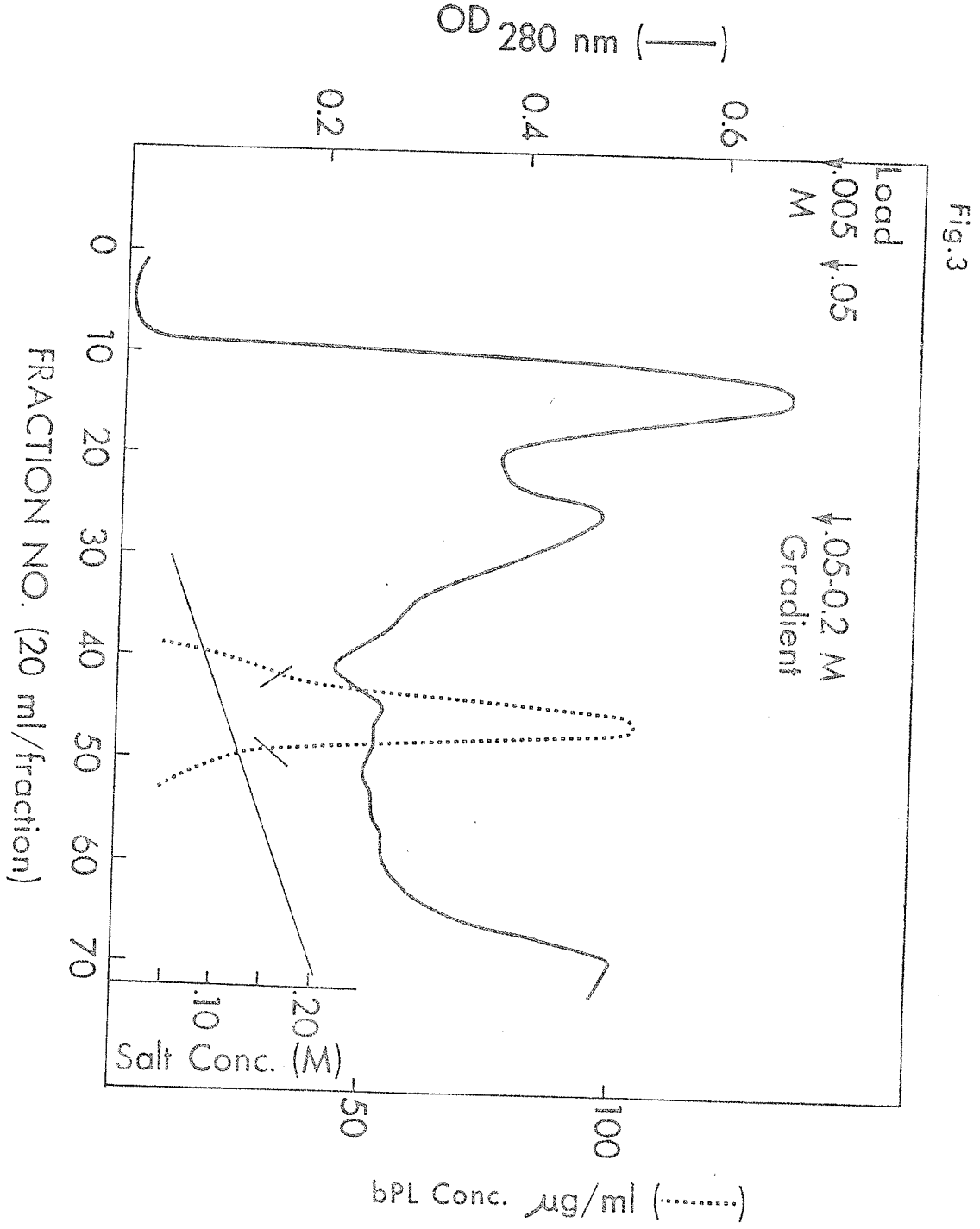

OD 280 nm (——)
0.2
0.4
0.6
Load
.005 M
.05
.05-0.2 M
Gradient
FRACTION NO. (20 ml/fraction)
0
10
20
30
40
50
60
70
.10
.20
Salt Conc. (M)
50
100
bPL Conc. µg/ml (.....)

Fig.3

Figure 4

SP-Sephadex chromatography of the receptor active pool after HTP chromatography. The ion exchange column (2X5 cm) was equilibrated with 0.005 M sodium phosphate buffer, pH 4.8. Following sample application, the column was washed with the 0.005 M buffer until protein elution, as determined by fraction optical density at 280 nm, was minimal and stable. Elution of bPL was accomplished by the stepwise application of a 0.05 M sodium phosphate buffer, pH 5.8.

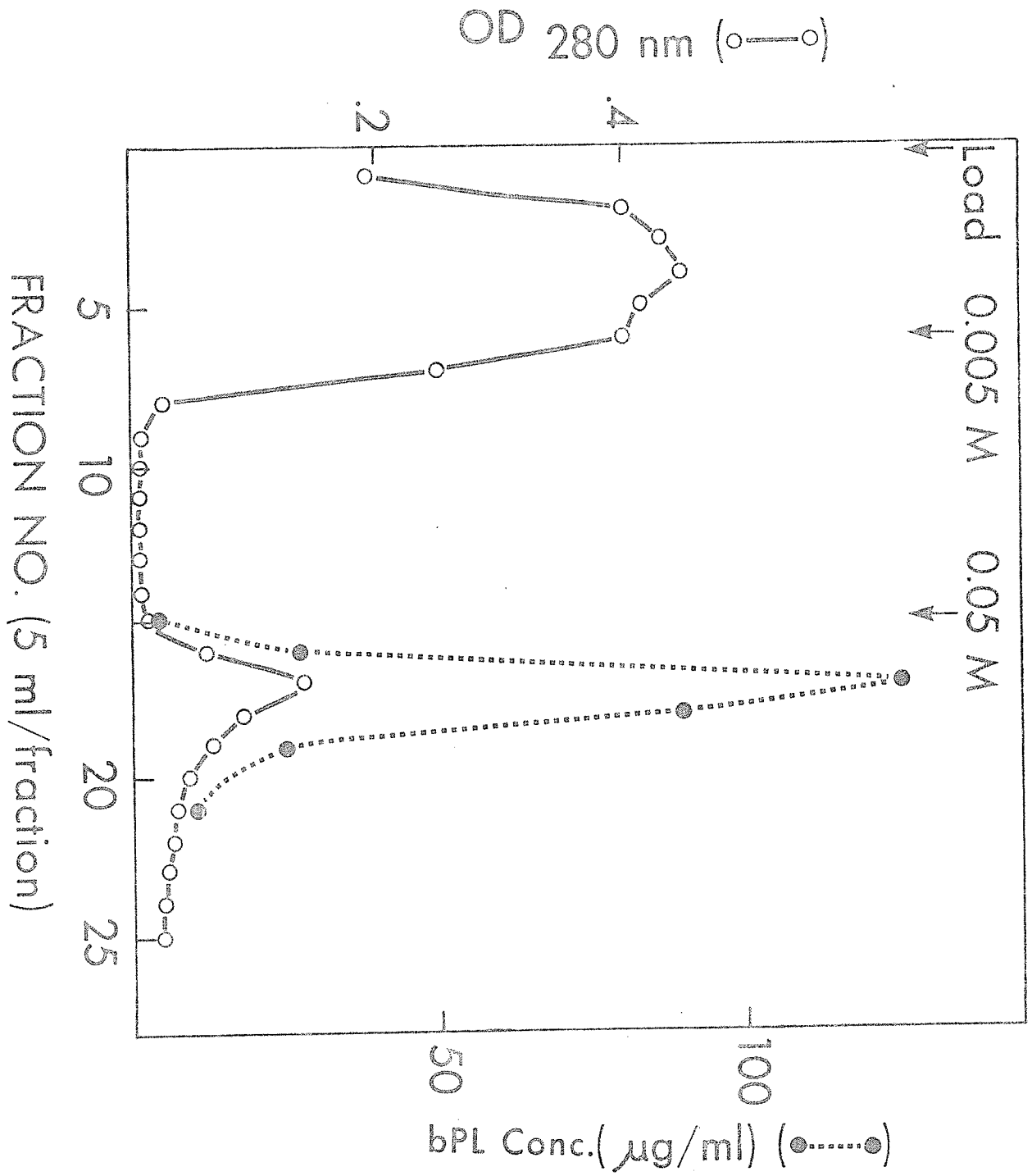


Fig. 4

against 0.005 M sodium phosphate buffer, pH 4.8.

SP - Sephadex Column Chromatography

The concentrated and dialyzed fractions eluted from the HTP column were applied to a SP- Sephadex column (Figure 4), equilibrated with 0.005 M sodium phosphate buffer, pH 4.8. The column was washed with the same buffer . bPL was eluted with a stepwise gradient of 0.05 M sodium phosphate buffer, pH 5.8.

The results of the various purification procedures are summarized in Table 1 and show how a total of 75 mg of bPL in 1 kg of scraped placental tissue is purified over 1000 fold to yield 1.5 mg of pure bPL.

B: PHYSICO-CHEMICAL CHARACTERIZATION OF bPL

All characterization of bPL was done on the purified product as described in Table 1.

1) Analytical Polyacrylamide Gel Electrophoresis

Figure 5 shows the protein staining pattern of the purified bPL, following acid (pH 4.3) gel electrophoresis. A single stained band is observed. A second gel containing bPL, electrophoresed in an identical manner at the same time, was sliced into consecutive gel segments and the slices eluted and assayed for bPL by RRA. All bPL was detected in the eluants from segments corresponding to the stained band.

2) Analytical Polyacrylamide Gel Isoelectric Focusing

Analysis of purified bPL by isoelectric focusing across a pH range of 3.5-9.5, is shown in Figure 6. The picture of the stained gel demonstrates that the bPL

TABLE 1

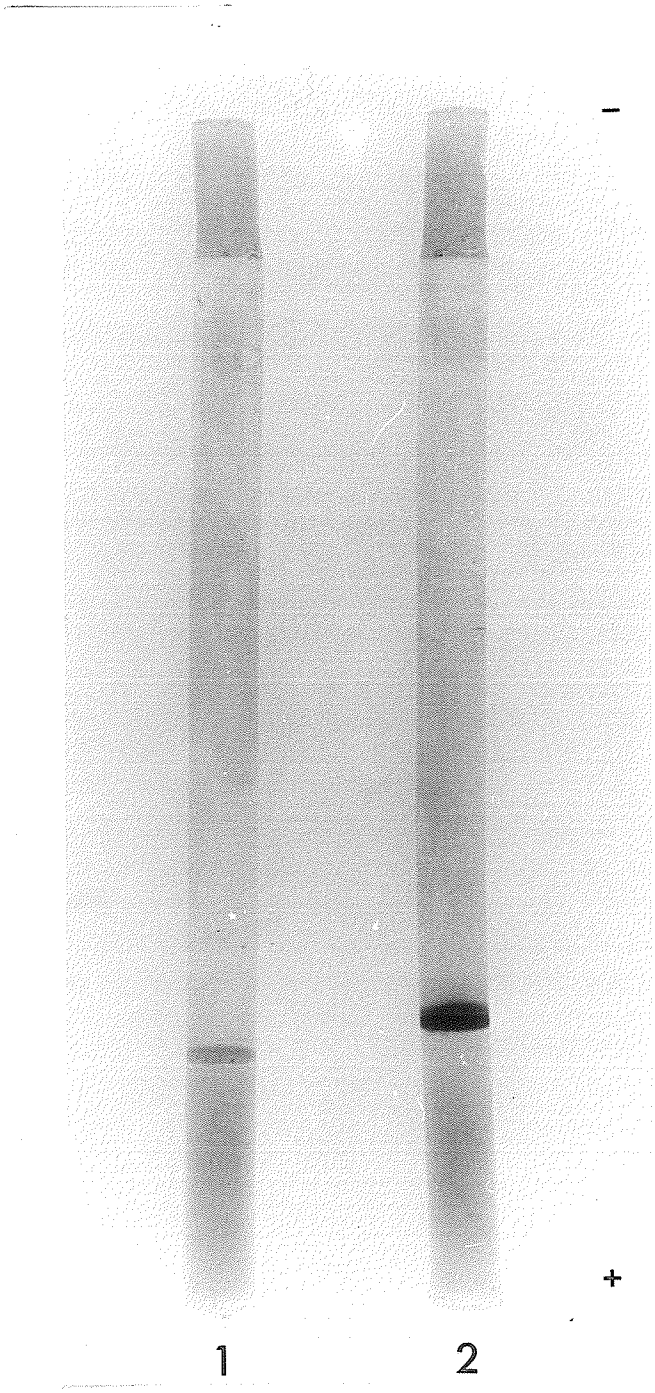
SUMMARY OF bPL PURIFICATION

	Total Volume	bPL Conc. (ug/ml)	Total bPL mgs	OD_{280}	μg bPL /OD unit	Recovery	Purification Factor
(1) Homogenate[1]	2000 ml	37.5	75.0	51.000	0.74	100 %	1.0
(2) 45-55% ASP	105 ml	280.0	29.4	126.000	2.22	39 %	3.0
(3) G-100 Effluent	105 ml	80.0	8.4	3.420	23.39	11 %	31.6
(4) HTP Effluent	100 ml	56.0	5.6	0.250	224.00	7.5 %	302.7
(5) SP-Sephadex Effluent	20 ml	74.0	1.5 *	0.085	882.35	2.0 %	1192.4

[1] Wt. of Starting Placental Tissue= 1 kg

*Total Protein Measured by Coomassie Blue Method = 2.5 mg

FIGURE 5

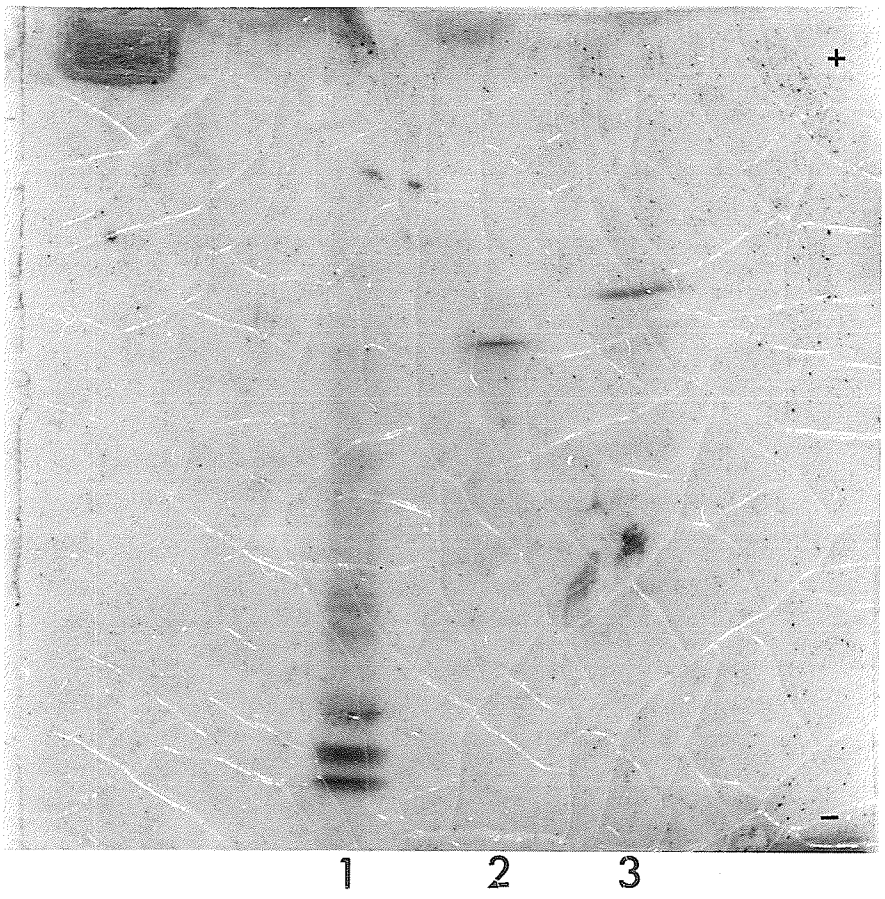


Blank leaf to correct numbering.

Figure 6

Polyacrylamide gel isoelectric focusing of bGH, bPRL, and bPL across pH range of 3.5-9.5. Lane 1 had 14 ug of bGH applied, lane 2 had 6 ug of bPRL applied, and lane 3 had 8 ug of bPL applied. bGH and bPRL were obtained from NIH (NIH-GH-B18 and NIH-P-B5 respectively) while bPL was purified as described. Eluates of gel slices obtained from an adjacent lane of bPL demonstrated activity in the RRA-hGH coincident with the stained band of bPL (not shown).

FIGURE 6



preparation consists primarily of a single, homogeneous protein band. An adjacent unstained channel of bPL was sliced and the consecutive segments eluted. Assay of the eluates by RRA verified that all the bPL corresponded to the major stained protein band with a pH of about 5.5 (not shown). Purified bPRL in this system also exhibits only one major protein band while pure bGH consists of three major protein components along with several less prominently stained constituents.

Figure 7 illustrates the distribution of bPL and proteins following isoelectric focusing in a Sephadex gel system in the absence of ampholytes, of an aliquot of the 45-55% ammonium sulfate fractions obtained during purification. While the great majority of protein focuses at a pH of 7, most of the bPL focuses at a pH of about 5.5 with smaller peaks at PI's of 5.3 and 5.8.

3) Analytical Sodium Dodecyl Sulfate (SDS) Polyacrylamide Slab Gel Electrophoresis of bPL

Figure 8 shows the SDS gel electrophoretic pattern of pure bPL after staining with Coomassie Brilliant Blue R-250. One stained protein band, migrating with an apparent molecular weight of 35,000 daltons, is seen. An adjacent lane of the SDS gel shown in this figure also had bPL applied but was not stained after electrophoresis. Instead, it was sliced into 3 mm wide sections and each section eluted overnight at $4^{o}C$ in 1 ml of 0.025 M Tris-HCl buffer, pH 7.6 containing 0.1% BSA. Assay of the eluates from these sections in the

Figure 7

Distribution of bPL and protein after isoelectric focusing in a Sephadex gel system without ampholytes, a 40-60% ammonium sulfate precipate of bovine placental extract. Protein focusing was followed by division of the gel into 30 lanes, measurement of pH profile (Δ) by surface pH electrode, and elution of the gel segments with distilled water. Aliquots of the eluants were then assayed in the RRA for hGH (O) and the protein estimated by measuring the optical density at 280 nm (OD_{280}; □). Each eluate was dialyzed overnight at $4^{o}C$ against distilled water. The dialysis was carried out to reduce the concentration of ampholytes in the samples prior to absorbance measurements.

Fig.7

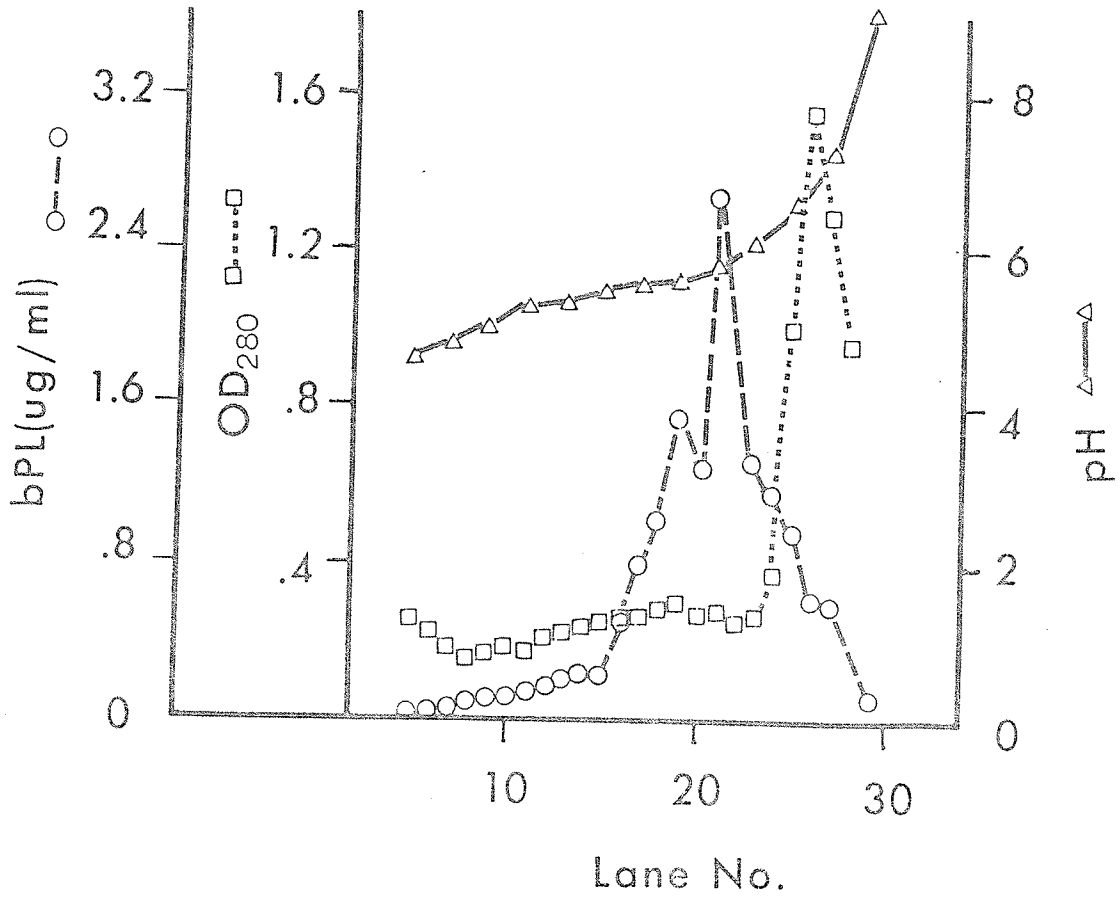
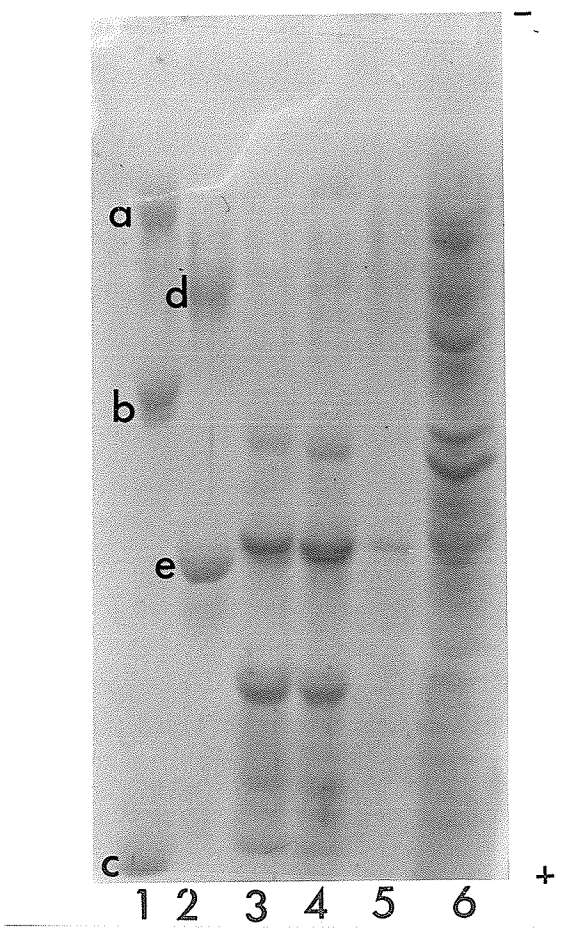


Figure 8

Protein staining pattern of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of bPL. All protein samples were heated in the presence of 2% 2-mercaptoethanol and 2% SDS prior to their application to the 12% gel.

Lane #	Sample Description			Molecular Weight
1	standards	a	phosphorylase a	95,000
		b	ovalbumin	45,000
		c	cytochrome c	13,000
2	standards	d	bovine serum albumin	67,000
		e	carbonic anhydrase	30,000
3,4	HTP pool from bPL purification (12 ug bPL)			
5	pure bPL (4 ug)			37,000
6	G-100 pool from bPL purification (2 ug bPL)			

FIGURE 8



growth hormone receptor assay (RRA-hGH) indicated a small, though detectable peak of bPL coincident with the stained protein band (not shown).

4) Sephadex G-100 Elution Profile of Iodinated bPL (^{125}I-bPL)

Figure 9 shows the distribution of radiolabelled bPL after gel filtration. The specific binding of the tracer to rabbit liver membranes approached 15%.

5) Analytical SDS Acrylamide Slab Gel Electrophoresis of ^{125}I-bPL

Figure 10 is a photograph of the radioautogram of ^{125}I-bPL following SDS gel electrophoresis. The one major radioactive band exhibits an apparent molecular weight in the range between the two radiolabelled standards, ovalbumin and carbonic anhydrase (45,000-30,000 daltons respectively).

6) Molecular Weight Determination of bPL

Figure 11 demonstrates a semi-logarithmic plot of the molecular weight of known protein standards as a function of their elution characteristics from a Sephadex G-100 gel exclusion column (Kav;1). The molecular weight of the iodinated forms of these standard proteins is also plotted as a function of their relative mobilities in an SDS polyacrylamide gel system (Rf;2). bPL elutes from the Sephadex gel with an apparent molecular weight of about 32,000 while the Rf of ^{125}I-bPL in the SDS gel is characteristic of a protein with a molecular weight of about 37,000.

Figure 9

The elution profile of ^{125}I-bPL after gel filtration upon Sephadex G-100 (geiger counts; O) and receptor binding ability (Δ) of ^{125}I-bPL. The ordinate represents the radioactive profile as measured by geiger counts and also denotes specific binding of ^{125}I-bPL to hGH receptors in the somatotropic RRA. Column dimensions were 2X50 cm and elution was carried out with PBS buffer at a flow rate of 60 ml/hr. The fractions eluted were 3 ml in volume and are represented on the abscissa.

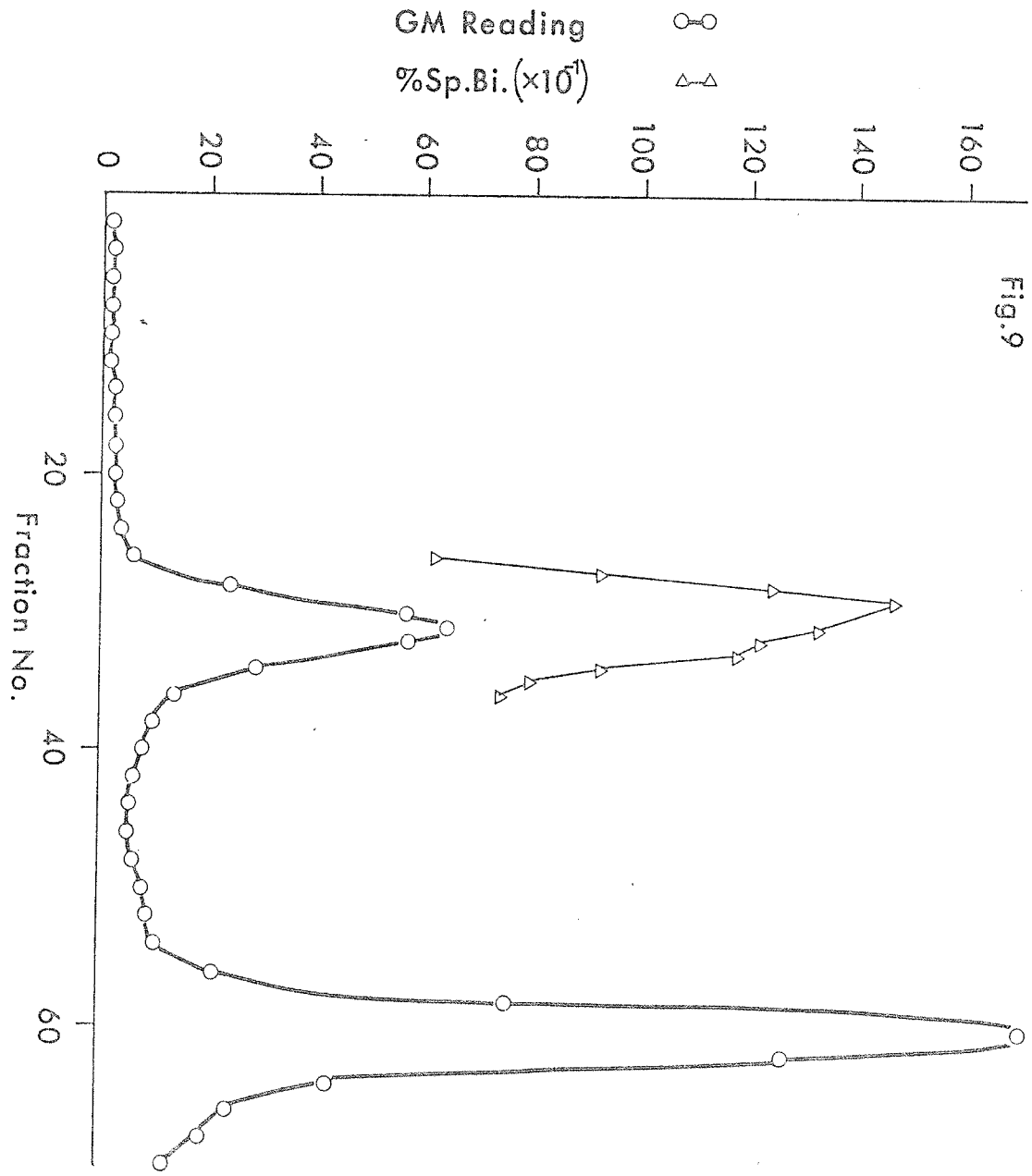

GM Reading
%Sp.Bi.(×10⁻¹)
0
20
40
60
80
100
120
140
160
Fraction No.
20
40
60

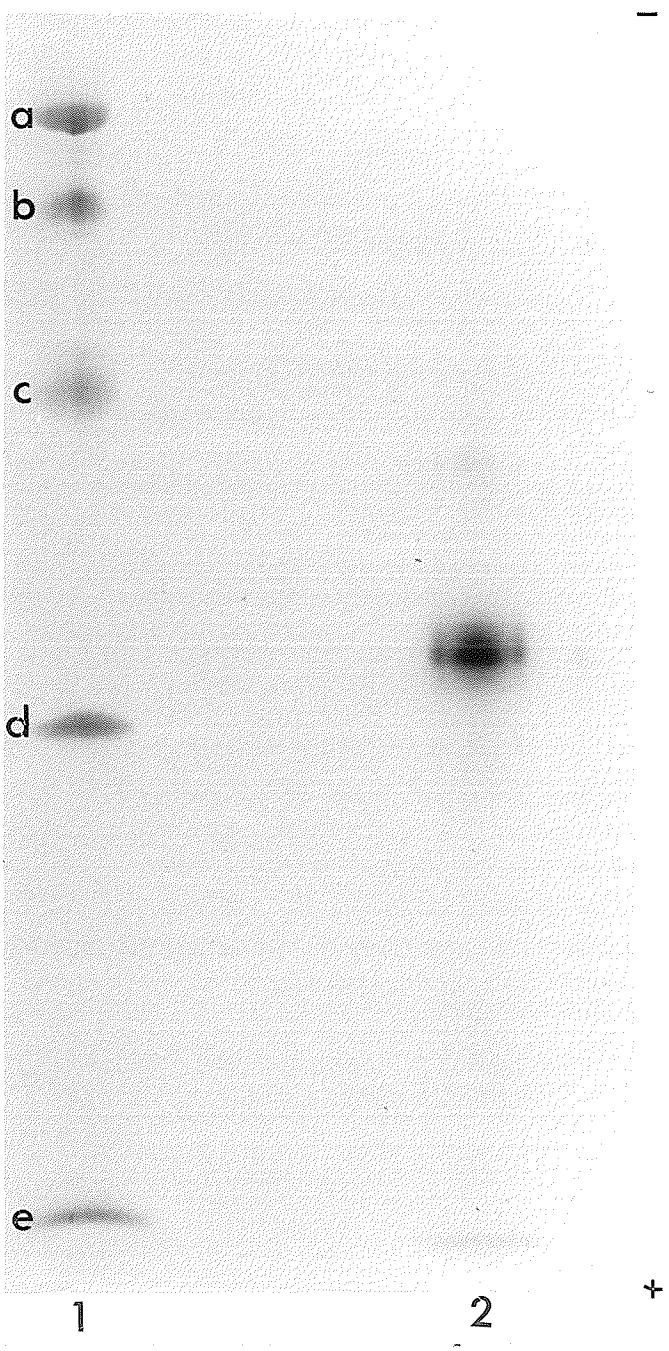
Fig.9

Figure 10

Sodium dodecyl sulfate polyacrylamide gel radioautography of ^{125}I-bPL.

lane 1	radioactive standards		molecular weights
	a	phosphorylase a	95,000
	b	bovine serum albumin	67,000
	c	ovalbumin	45,000
	d	carbonic anhydrase	30,000
	e	cytochrome c	13,000
lane 2	^{125}I-bPL		

FIGURE 10



C: IMMUNOLOGIC CHARACTERIZATION OF bPL

The ability of excess antisera, generated in either rabbits or guinea pigs to lactogenic and somatotropic hormones from several mammalian species, to precipitate ^{125}I-bPL was tested and results are shown in Table 2. Of all the antisera tested, only one generated to ovine placental lactogen (oPL) could precipitate a significant amount of the tracer. One antiserum to oPRL demonstrated minor tracer immunoprecipitation. Antiserum to bPL could only precipitate 56% of the added ^{125}I-bPL in this system.

D: IMMUNOHISTOCHEMICAL VISUALIZATION OF ANTI-bPL REACTIVE SITES IN FETAL BOVINE PLACENTAL TISSUE

The four photographs (A-D) of Figure 12 present a pictorial summary of the study designed to determine the presence of bPL within cells of the cryostat sectioned bovine cotyledon. All tissue section photographs presented in this figure were obtained from placental tissue at the seventh month of gestation. The portion of the placenta studied consisted entirely of fetal cotyledonary tissue from a single placetome and all sections are serial sections obtained from the same frozen tissue block.

Frame A shows the presence of intense brown staining throughout the cytotrophoblastic tissue denoting the presence of many anti-bPL reactive cellular sites. The arrow points out one of the characteristic binucleated cells seen in ruminant placental tissue which

Figure 11

The molecular weight determination of bPL based on a) the relative mobility (Rf) of bPL upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1); and b) the characteristic elution (Kav) of ^{125}I-bPL from a 2X50 cm column of Sephadex G-100 (2).

Fig.11

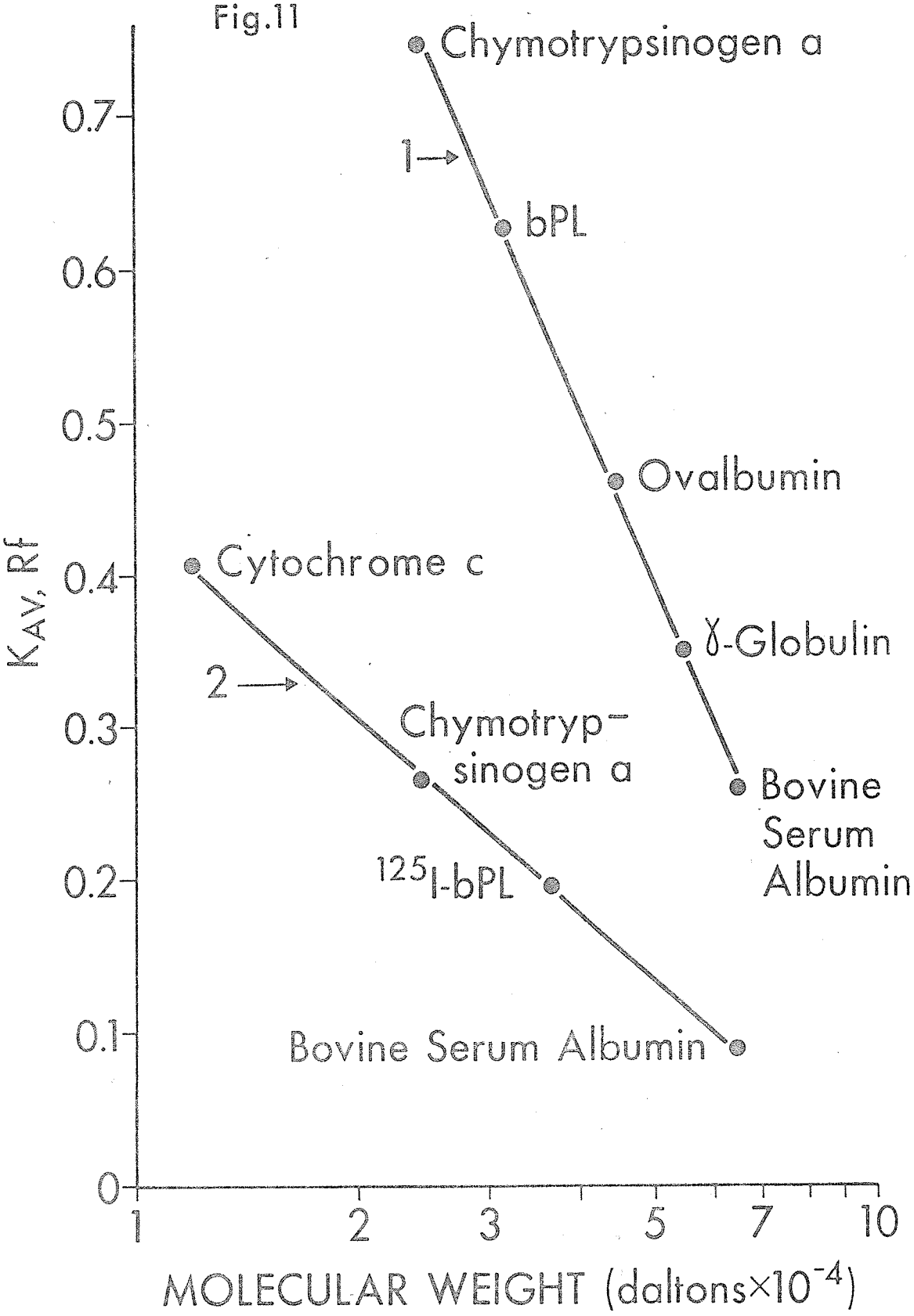

Chymotrypsinogen a
1
bPL
Ovalbumin
γ-Globulin
Bovine Serum Albumin
Cytochrome c
2
Chymotryp-sinogen a
125I-bPL
Bovine Serum Albumin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
KAV, Rf
1
2
3
5
7
10
MOLECULAR WEIGHT (daltons×10-4)

TABLE 2

MAXIMUM PRECIPITATION OF ^{125}I-bPL BY HOMOLOGOUS AND HETEROLOGOUS ANTISERA

ANTISERUM[1,2]	MAXIMUM TRACER PRECIPITATION (%)
A-bPL (B)	56.2
A-oPL (3-5)	36.3
A-hPL (DR 2-7)	0.6
A-rPL (C)	0
A-bPRL (45)	0.6
A-oPRL (FR 1-5)	4.7
A-hPRL (AR 26-7)	0.8
A-rPRL (A)	0
A-pPRL (#3)	0.4
A-oGH (1-3)	0.4
A-hGH (582-6)	0
NRS	0
NGPS	0

[1]WHERE A: ANTISERUM TO
b: BOVINE
o: OVINE
h: HUMAN
r: RAT
p: PORCINE

PL: PLACENTAL LACTOGEN
PRL: PROLACTIN
GH: GROWTH HORMONE
NRS: NORMAL RABBIT SERUM
NGPS: NORMAL GUINEA PIG SERUM

[2]ALL ANTISERA AT 1:10

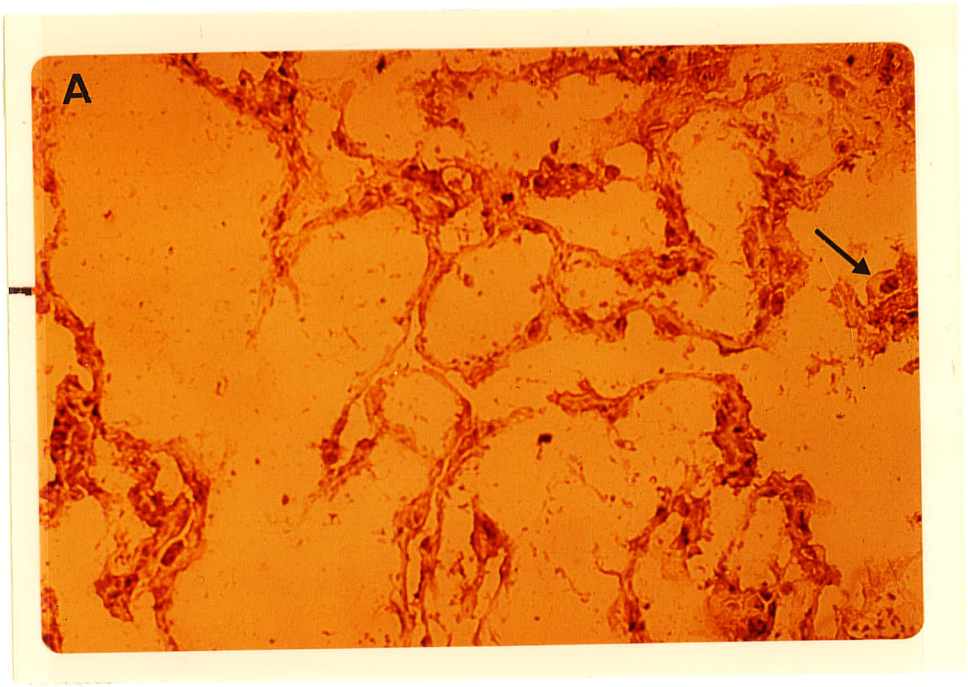
Figure 12

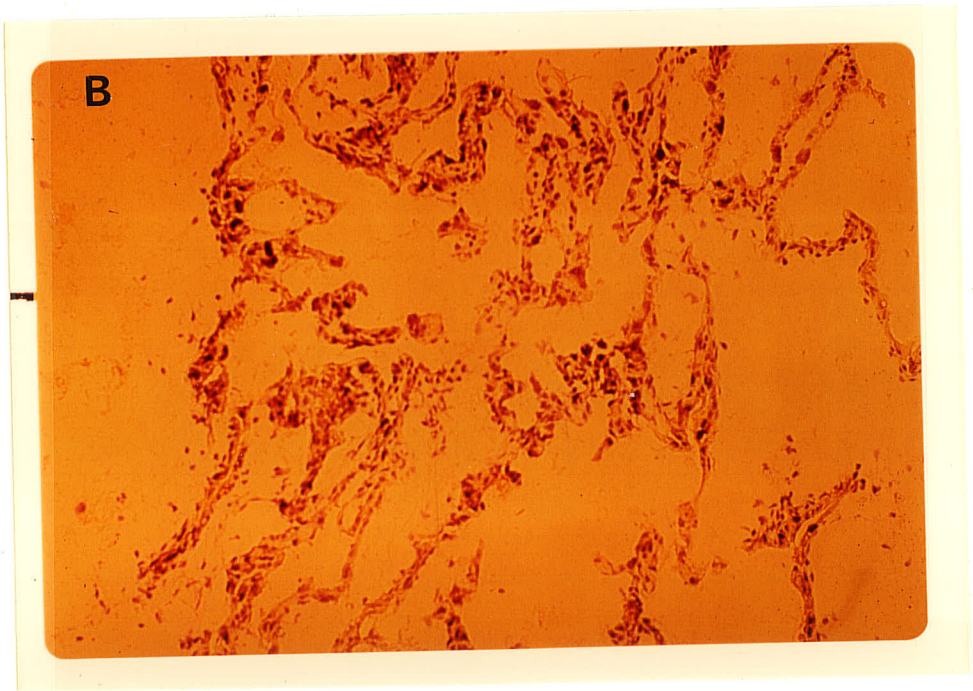
Immunohistochemical localization of anti-bPL reactive sites in fetal bovine cotyledons. Cryostat sections (5 u thick) of flash frozen placental tissue were slide mounted, fixed, and incubated in the presence of either anti-bPL, -bPRL, or -oGH antiserum. Following incubation and washing, the placental sections were treated with second antibody, then a soluble peroxidase-anti-peroxidase mixture followed by an exposure to a H_2O_2-diaminobenzoic acid mixture and finally counterstained with cresyl violet (as described in Materials and Methods).

A. Placental section treated with anti-bPL. The brown color represents the positive reaction of the oxidased, precipitated stain. The arrow denotes the presence of one of several stained binucleated cells in the cytotrophoblast of the fetal cotyledon.

B. Stained appearance of a placental section after treatment with anti-bPL previously incubated in the presence of excess bPL. Note the virtual absence of any brown stain.

FIGURE 12





has been stained.

Frame B shows the virtual absence of any of the obvious staining, as shown in Frame A, when the primary anti-bPL antiserum was preincubated in the presence of excess bPL prior to its application fo the section.

Picture C demonstrates the ineffectiveness of excess bPRL (and bGH; not shown), to neutralize the stain-intensity of the primary antiserum when the anti-bPL was first incubated in the presence of an excess of these bovine pituitary hormones.

Photograph D presents the lack of any staining reaction in the placenta when the primary antiserum employed was anti-bPRL. Anti-oGH antiserum and normal rabbit serum yielded identical results.

E: STUDIES ON THE BIOSYNTHESIS OF bPL BY FETAL BOVINE PLACENTAL TISSUE in vitro

Table 3 indicates the total amount of radioactive material in the placental homogenate and incubation medium which could be immunoprecipitated by antisera to bPL, oPL, bPRL, and oGH.

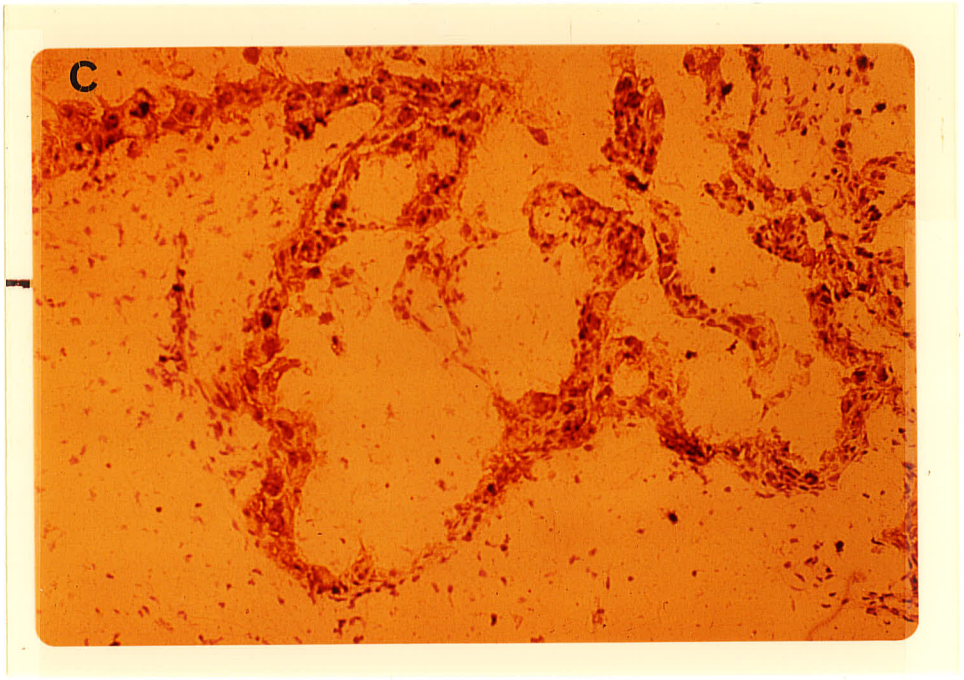
Figure 13 presents a picture of the fluorogram developed from the SDS acrylamide gel to which had been applied aliquots of the redissolved immunoprecipitates obtained from the placental tissue homogenate. The arrow in lane 7 of the gel denotes the unique, newly synthesized, radiolabelled component specifically precipitated by anti-bPL antiserum. It has migrated the

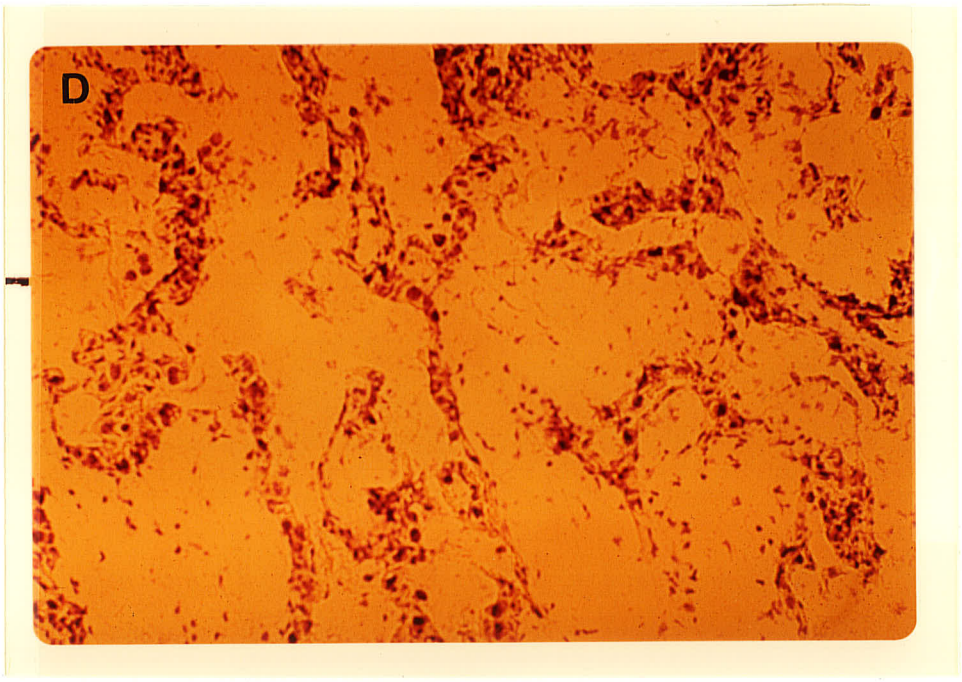
Figure 12

C. Staining of a placental section after treatment with anti-bPL previously incubated in the presence of 5 ug bPRL. The positive staining intensity is unaffected by the pretreatment of the antiserum. The same result was obtained when anti-bPL antiserum was preincubated with 5 ug bGH (not shown).

D. Lack of any positive staining seen when placental section was processed using anti-bPRL antiserum as the first antibody. Similar results were obtained when the first antibody was anti-oGH antiserum or normal rabbit serum (not shown).

FIGURE 12





same distance in the gel as the marker ^{125}I-bPL in lane 10.

F: DEVELOPMENT AND APPLICATION OF AN HOMOLOGOUS RADIOIMMUNOASSAY FOR bPL

1) Standard Curve of bPL in the Radioimmunoassay (RIA)

Figure 14 illustrates the standard curve of bPL in the RIA. The assay sensitivity is 20 ng/ml with a final antiserum concentration of 1:10,000. Dilutions of a crude placental extract produce a displacement curve which parallels the standard curve. Pregnant serum, obtained from a slaughtered cow in the seventh month of pregnancy, shows a non-parallel displacement curve when compared to bPL standards. Partial displacement curves with anterior pituitary extract and non-pregnant cow (not shown) and steer serum also suggest a condition of non-parallelism to the bPL standards exists for them. Purified bGH, bPRL, hGH, hPRL, and oPRL show no cross-reaction in the assay at 1000 ng/ml (not shown).

2) Assay of Bovine Fluid Samples in the Homologous RIA for bPL.

Table 4 summarizes the results obtained when measuring immunoactivity employing the RIA for bPL. Steer serum samples and non-pregnant cow serum and milk samples all exhibited apparent bPL immunoactivity. While pregnant cow and fetal serum samples had higher levels of this bPL activity than non-pregnant cows, no correlation was seen between these values and gestational age during the period of pregnancy tested.

TABLE 3

^{35}S-METHIONINE RADIOLABELLED IMMUNOPRECIPITATES OF BOVINE PLACENTA

ANTISERUM	IMMUNOPRECIPITATED COUNTS (CPM) PLACENTAL HOMOGENATE	INCUBATION MEDIUM
ANTI-bPL	24619	3867
ANTI-oPL[1]	19673	2398
ANTI-oGH[2]	16319	1735
ANTI-bPRL[3]	15153	1785
NRS[4]	13008	2163

[1,2,3] ANTIBODIES TO OVINE PLACENTAL LACTOGEN, OVINE GROWTH HORMONE AND BOVINE PROLACTIN RESPECTIVELY.

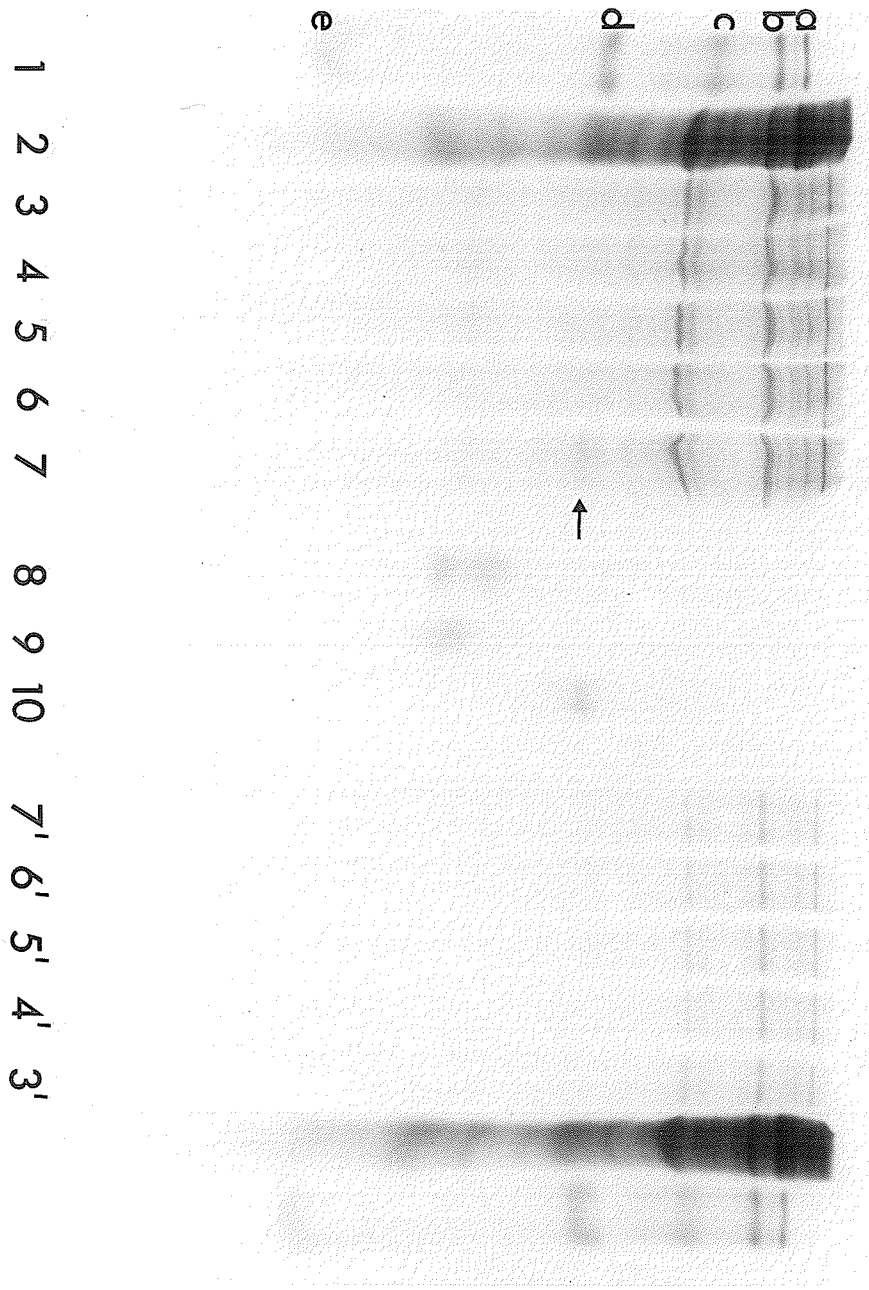
[4] NORMAL RABBIT SERUM

Figure 13

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic fluorogram of ^{35}S-methionine radiolabelled, immunoprecipitated placental proteins. Fetal bovine placental tissue was incubated at 37°C for 6 hours in the presence of ^{35}S-methionine. The tissue was then homogenized, centrifuged at 100,000 x g and aliquots of the supernatant incubated with normal rabbit serum (NRS) and antisera to various bovine and ovine hormones. The antisera employed were tested at two different concentrations (1 ul and 5 ul). The antibody-radiolabelled protein complexes were precipitated with Staph A proteins. These precipitates were washed, boiled in the presence of 2% SDS and 2% 2-mercaptoethanol and applied to the SDS gel. Lane numbers with superscripts represent immunocomplexes formed when the 1 ul volume of antiserum was used. Identically numbered lanes without superscripts represent immunocomplexes formed using the 5 ul volume of antiserum.

lane 1	standards (as for Figure 10)
lane 2	whole placental homogenate
lanes 3,3'	NRS and placental homogenate
lanes 4,4'	anti-bPRL and placental homogenate
lanes 5,5'	anti-oGH " " "
lanes 6,6'	anti-oPL " " "
lanes 7,7'	anti-bPL " " "
lane 8	^{125}I-bGH
lane 9	^{125}I-bPRL
lane 10	^{125}I-bPL

FIGURE 13



Leaf blank to correct numbering.

Figure 14

The standard curve of the homologous RIA for bPL (●). Comparison of dilution curves for bovine placental extract (△), pregnant cow serum (■), bovine anterior pituitary extract (O), and steer serum (▲) is presented. Purified bPRL, bGH, bPRL, hGH, oPRL, and oPL, showed no cross-reaction at concentrations of 1000 ng/ml (not shown).

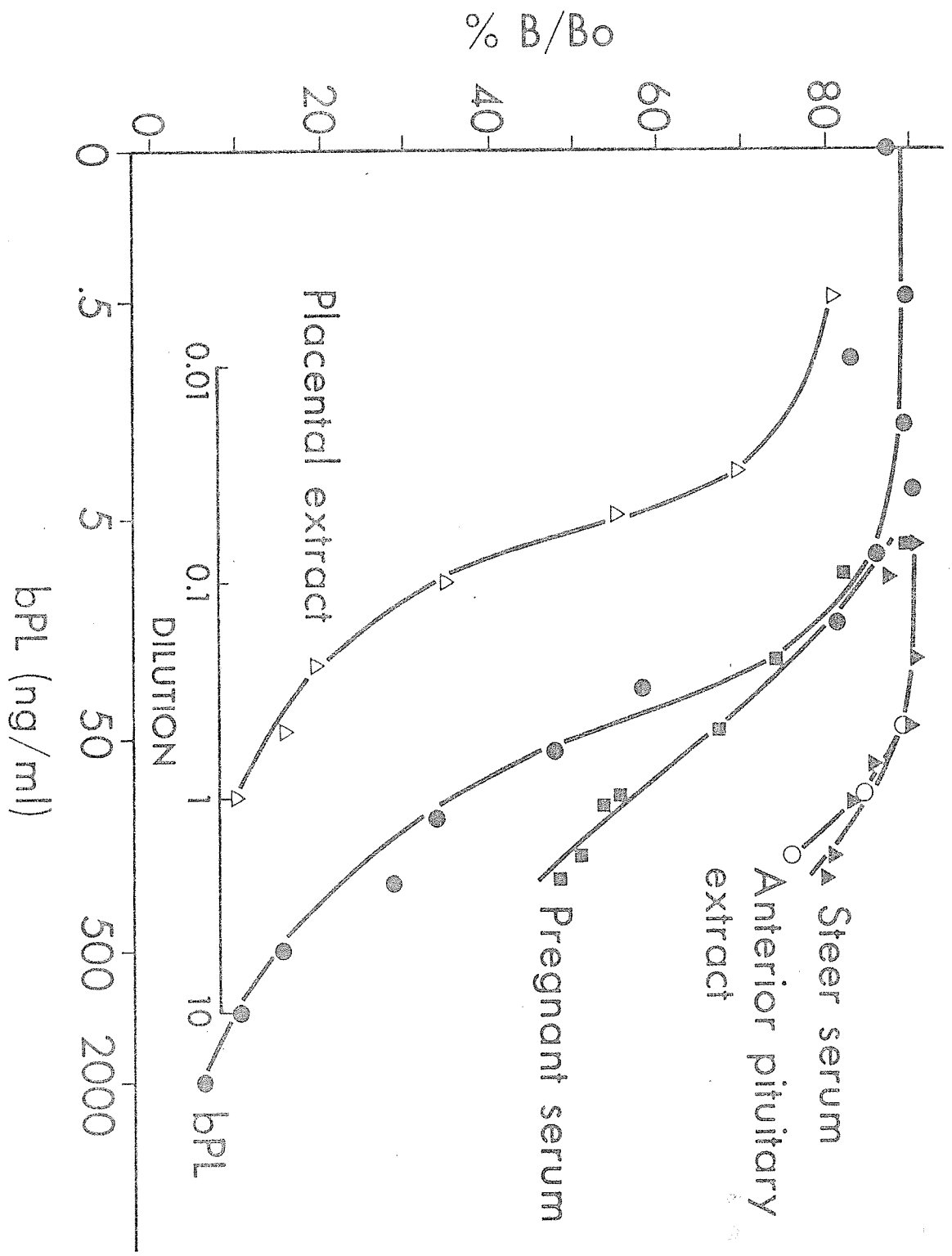

% B/Bo
0
20
40
60
80
0
.5
5
50
500
2000
bPL (ng/ml)
0.01
0.1
1
10
DILUTION
bPL
Placental extract
Pregnant serum
Anterior pituitary extract
Steer serum

Fig.14

TABLE 4

'bPL' IN BOVINE MILK AND SERUM DETERMINED BY HOMOLOGOUS RADIOIMMUNOASSAY FOR bPL (RIA-bPL)

SAMPLE		RIA-bPL (NG/ML) RANGE	RIA-bPL (NG/ML) MEAN	GESTATIONAL AGE RANGE (DAYS)
STEER	SERUM	24-45	35	
NON-PREGNANT COW	SERUM	20-49	32	
	MILK*	57-58	58	
PREGNANT COW	SERUM	73-125	98	60-200
	MILK	27-60	43	70-206
FETAL	SERUM	49-84	63	135-210

* TWO SAMPLES

G: CHARACTERIZATION OF THE COMPONENTS COMPRISING THE HOMOLOGOUS RIA FOR bPL

(Why is the RIA Non-functional?)

The studies to be described were designed to add the elements of sensitivity and specificity to the RIA for bPL. The examination for a cause of the faulty RIA involved the evaluation of 1) the antibody specificity, 2) the purity of the tracer (to detect displacement of an iodinated contaminant), 3) the possibility of a bPL-binding protein in bovine serum which, if present, would bind tracer during the RIA incubation and yield spuriously elvated levels of bPL, and 4) pregnant cow serum to see how many, if any, unique constituents were measured in the RIA.

1) Studies Related to the Antiserum Raised Against bPL

Figure 15 presents the picture of the protein staining patterns of bPL and bPRL after isoelectric focusing in a ployacrylamide gel over a pH range of 3.5-9.5. An adjacent unstained lane of bPL, in which consecutively sliced segments were eluted and assayed in the RRA for hGH and in the RIA for bPL, confirmed that the only peak of hGH-like activity and the largest portion of immunoactivity corresponded to the major stained protein band at pH 5.5. A minor cross-reacting component of the bPL preparation was eluted at about pH 6.0.

Eluted bPL, detected in the receptor and immunoassays, is depicted in the figure beneath the photograph.

Figure 16 (A and B) is a pictorial representation

Figure 15

Polyacrylamide gel isoelectric focusing pattern of bPL and bPRL across a pH range of 3.5-9.5. Both hormones were applied to wicks at the cathodal end of the gel. Focusing was followed by the staining of a portion of the gel as shown in the figure while an adjacent lane of bPL was sliced into 20 segments. These segments were eluted overnight at $4^{o}C$ in 1 ml 0.1% BSA-Tris-HCl buffer, pH 7.6 containing 10 mM $MgCl_2$. Aliquots of the buffer were assayed in the RRA for hGH and in the RIA for bPL on the following day. hGH equivalents and immunoactive bPL levels in ng/ml are indicated for the eluted slices corresponding to their location in the gel. See next page.

FIGURE 15

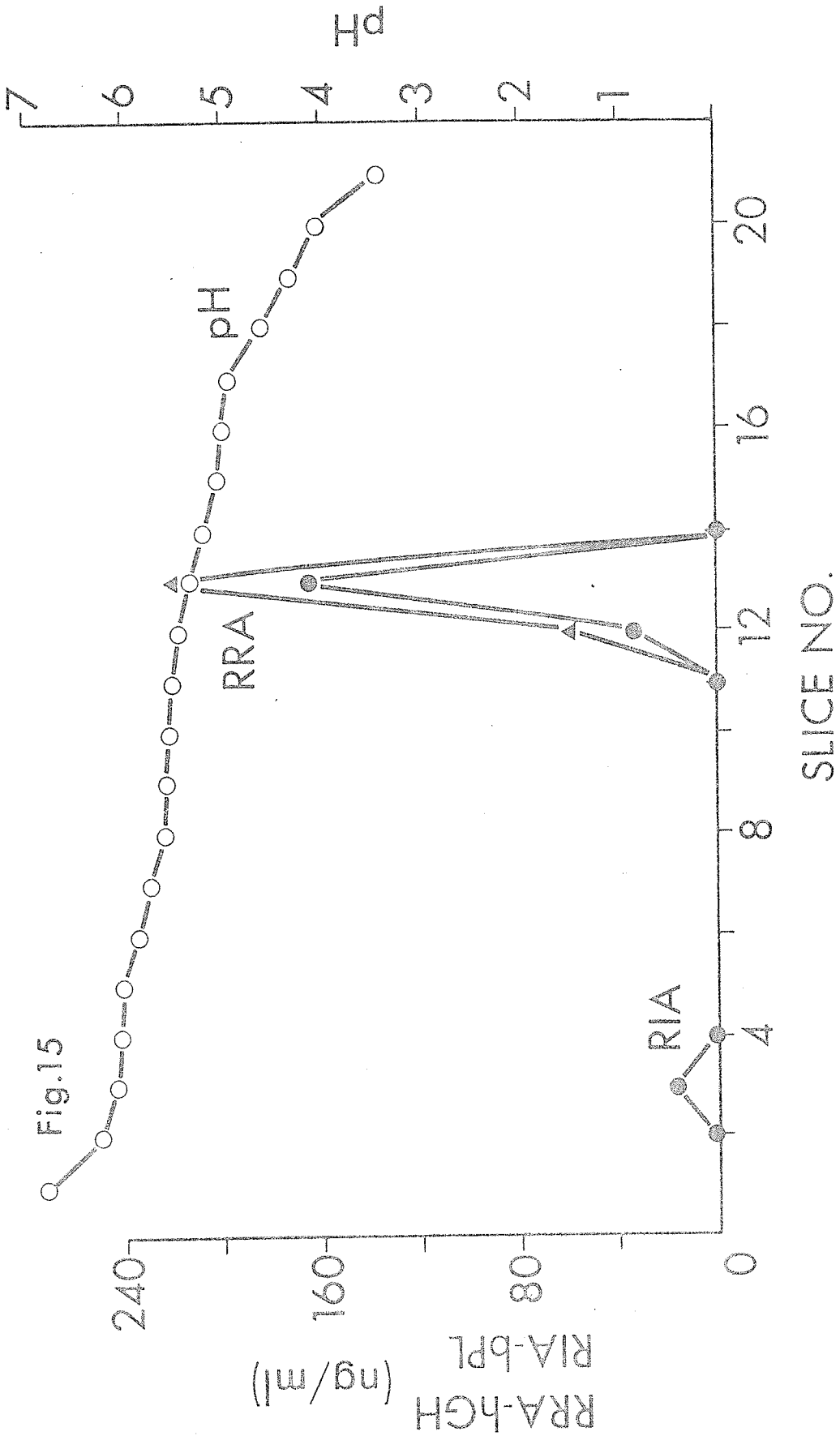

pH
7
6
5
4
3
2
1
pH
RRA
RIA
20
16
12
8
4
0
SLICE NO.
240
160
80
0
RRA-hGH
RIA-bPL
(ng/ml)

Fig.15

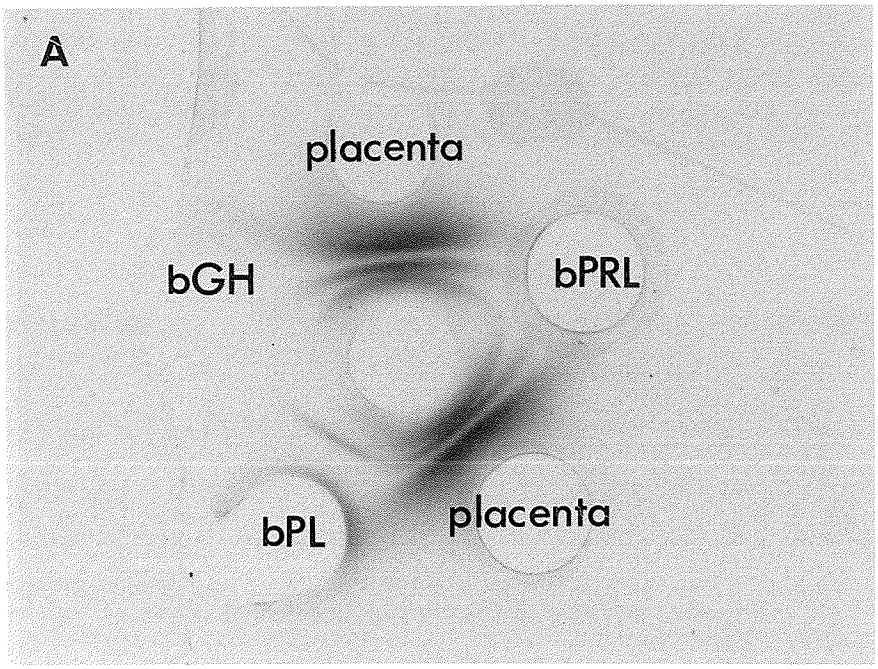
Figure 16

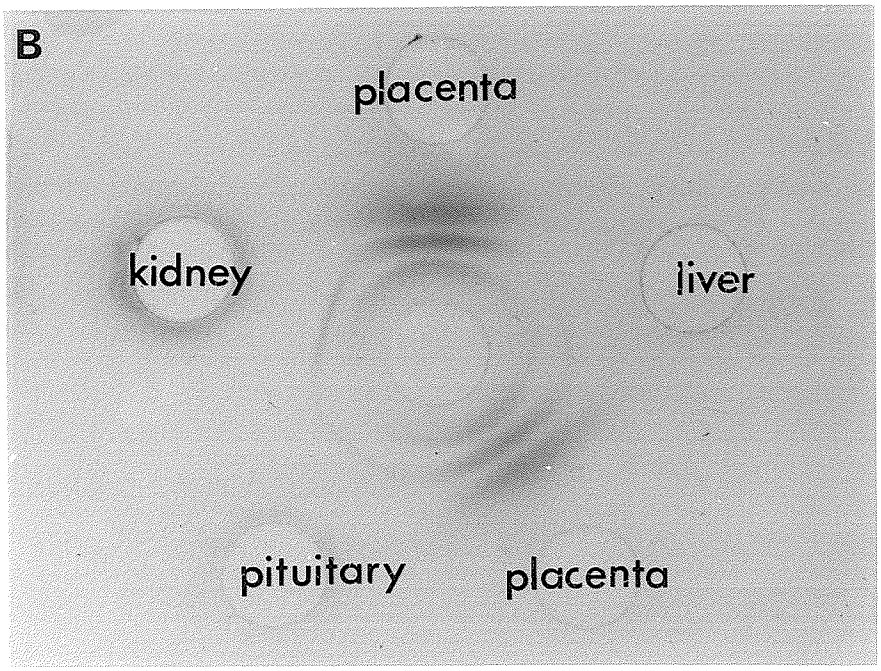
Staining pattern of immunoprecipition lines formed between antiserum to bPL and various bovine tissue extracts and purified hormones in an Ouchterlony double immunodiffusion system.

A. Coomassie blue stained precipitin lines between antiserum to bPL and bovine placental extract and bPL. No immune complex is seen between the antiserum and bPRL or bGH.

B. Stained precipitin lines are seen between the antiserum and all tissue extracts tested here.

FIGURE 16


A
placenta
bGH
bPRL
bPL
placenta


B
placenta
kidney
liver
pituitary
placenta

of results obtained employing the antiserum to bPL in the Ouchterlony immunodiffusion system. The antiserum in the center well of each gel shows at least three prominently stained preciptin lines with antigens present in the placental extracts of both gel A and B. The middle band of immunoprecipitation reaction with the placenta in gel A forms a continuous line with the immune complex formed between the antibody and purified bPL. Gel A shows that neither bGH nor bPRL contains components immunoprecipitable by the antiserum. Gel B demonstrates that a common antigenic component of extracts from the placenta, pituitary, kidney, and liver forms a continuous line of precipitation with the polyclonal antiserum.

2) Aspects of ^{125}I-bPL Immunoprecipitation by Anti-bPL Antiserum

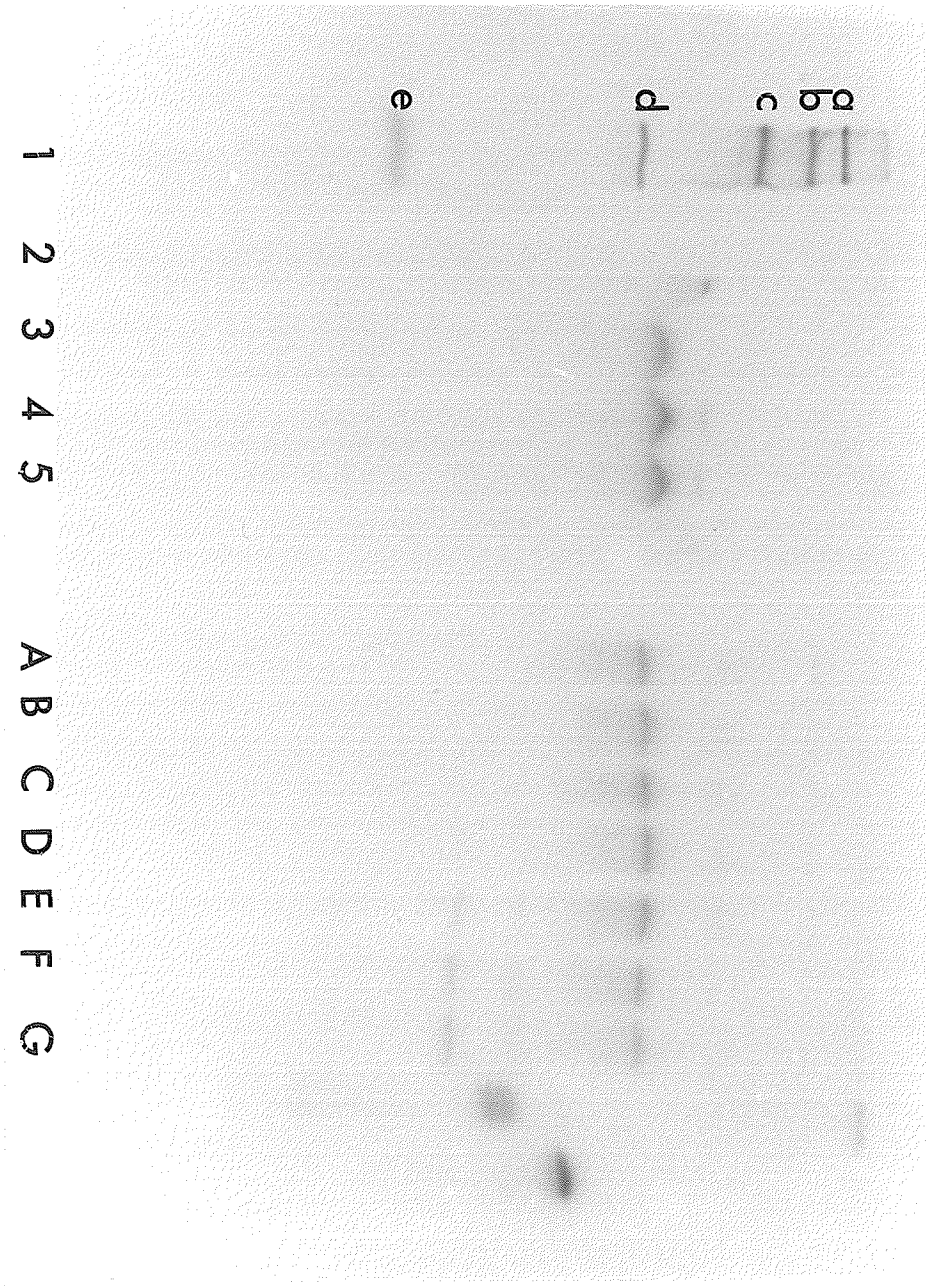
Figure 17 pictures the radioautogram of an SDS acrylamide gel following electrophoresis of applied ^{125}I-bPL previously immunoprecipitated by either normal rabbit serum (NRS), antibodies to oPL (a-oPL), or antibodies to bPL (a-bPL). Lanes A to G represent the precipitates of individual fractions of ^{125}I-bPL following Sephadex G-100 chromatography. A pool of these fractions was then precipitated with NRS (lane 2), a-oPL (lane 3), and a-bPL (lanes 4,5). The a-bPL precipitate consists of a major radioactive component at about 35,000 daltons with a minor band at a slightly higher molecular weight (in the range of the tracer component

Figure 17

Sodium dodecyl sulfate radioautogram of immunoprecipitated ^{125}I-bPL. ^{125}I-bPL was incubated overnight at 4^{o}C in the presence of either normal rabbit serum (NRS), or antiserum to ovine PL (a-oPL), or antiserum to bovine PL (a-bPL). The tracer-antibody complex was precipitated with a second antibody to rabbit gamma globulin. The immune complex was dissociated in 2% SDS and 2% 2-mercaptoethanol with heat and applied to the SDS gel.

lane 1	standards (as for Figure 10)
lane 2	^{125}I-bPL pool and NRS
lane 3	^{125}I-bPL pool and a-oPL
lanes 4,5	^{125}I-bPL pool and a-bPL
lanes A-G	individual fractions of ^{125}I-bPL pool (following tracer elution on Sephadex G-100) and a-bPL

FIGURE 17



precipitated non-specifically by NRS). The a-oPL antiserum appeared to precipitate only a 35,000 radiolabeled band.

3) Examination of Bovine Serum for the Possible Existence of a Binding Protein for bPL

The radioactive elution profile of ^{125}I-bPL from a Sephadex G-100 column (2X50 cm) following a preincubation of the tracer in either PBS buffer (A) or third trimester pregnant cow serum (B) is shown in Figure 18. The overall profile is unaffected by incubation of the tracer in serum. The decreased total radioactivity in profile B is due to the fact that only one-quarter of the amount of tracer chromatographed in profile A was added to the pregnant serum.

Figure 19 illustrates the effect of a preincubation in either PBS buffer or pregnant serum on the receptor activity of purified bPL following chromatography on a Sephadex G-100 column. The displacement of ^{125}I-hGH in the RRA for hGH by individual eluted fractions of bPL was essentially identical for either tested condition.

4) Application of Biochemical Techniques to Search Endogenous bPL

Figure 20 illustrates the eluted bPL immuno-activity present in bovine placental extract, pregnant cow serum (second trimester), and pure bPL following isoelectric focusing in an acrylamide gel across a pH range of 4-6.5. A peak of ^{125}I-bPL displacement in the

Figure 18

Gel filtration elution profile of ^{125}I-bPL following a 6 h preincubation in either 5 ml of PBS buffer (A) or 5 ml of pregnant cow serum (B). The Sephadex G-100 used for the chromatographic separation of radioactive components comprising the bPL tracer was packed in a 2X50 cm column. In both cases the tracer was eluted using PBS buffer at a flow rate of 55 ml/hr. The fraction number and volume is indicated on the abscissa while the ordinate denotes eluted radioactivity in cpm.

A. Total radioactivity applied to the column was 3.2×10^7 cpm. The recovery of radioactivity was 59%.

B. Total radioactivity applied to the column was 6.4×10^6 cpm. The recovery of radioactivity was 89%.

Fig.18

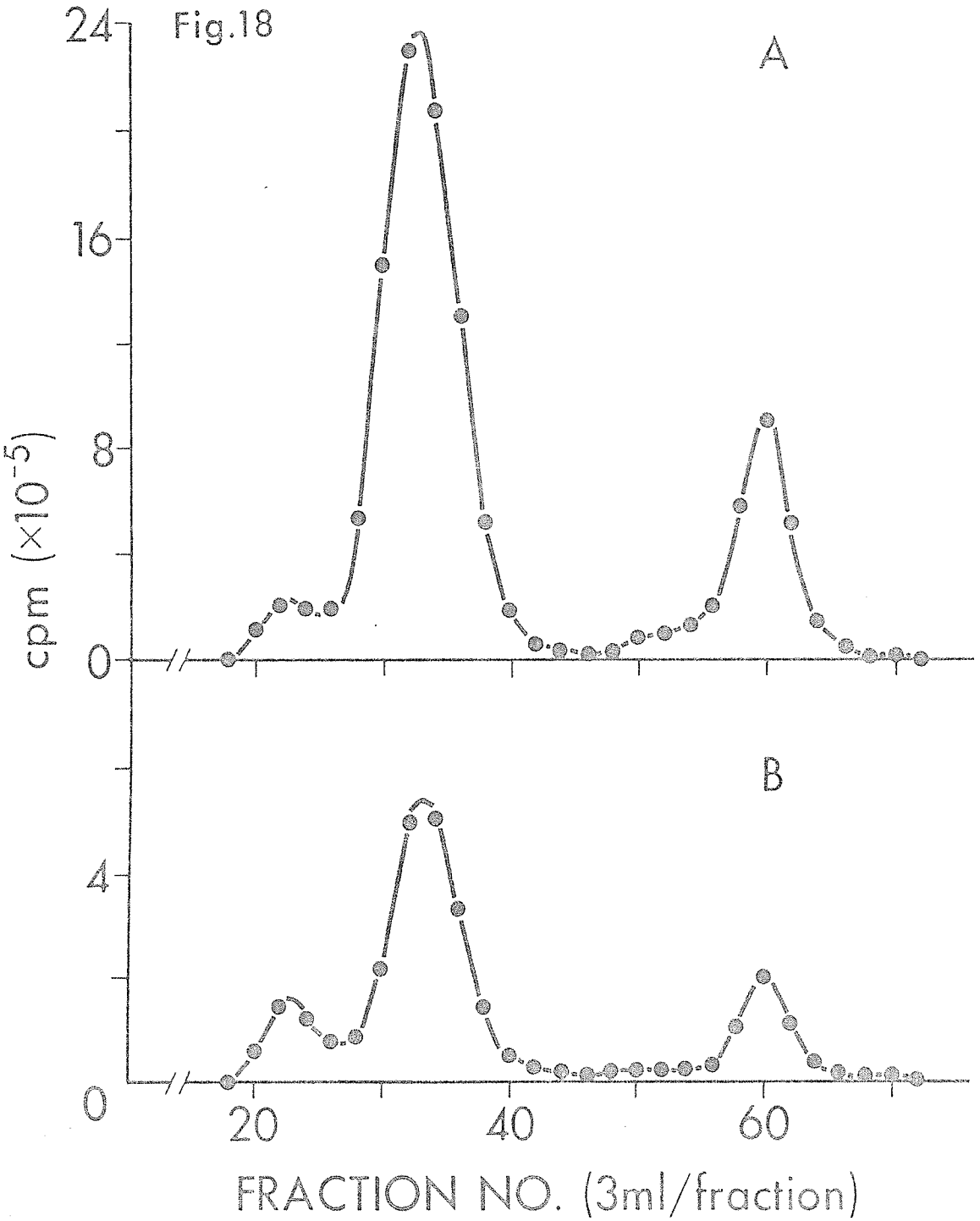


Figure 19

The Sephadex G-100 elution profile of bPL previously incubated for 6 h at $4^{o}C$ in either 5 ml of PBS buffer (●) or 5 ml of pregnant cow serum (▲). The gel filtration column (2X50 cm) was eluted with PBS buffer at a flow rate of 55 ml/hr following the separate application of either the preincubated 5 ml buffer-bPL mixture or 5 ml pregnant serum-bPL mixture. ^{125}I-hGH counts bound to the rabbit liver membranes in the presence of the eluted fractions of bPL are indicated on the ordinate. The total amount of bPL applied in PBS buffer was 122 ug with a final recovery of 95 ug (78% recovery). The amount of bPL applied in the pregnant serum sample was 68 ug with a recovery of 59 ug (86% recovery).

Fig.19

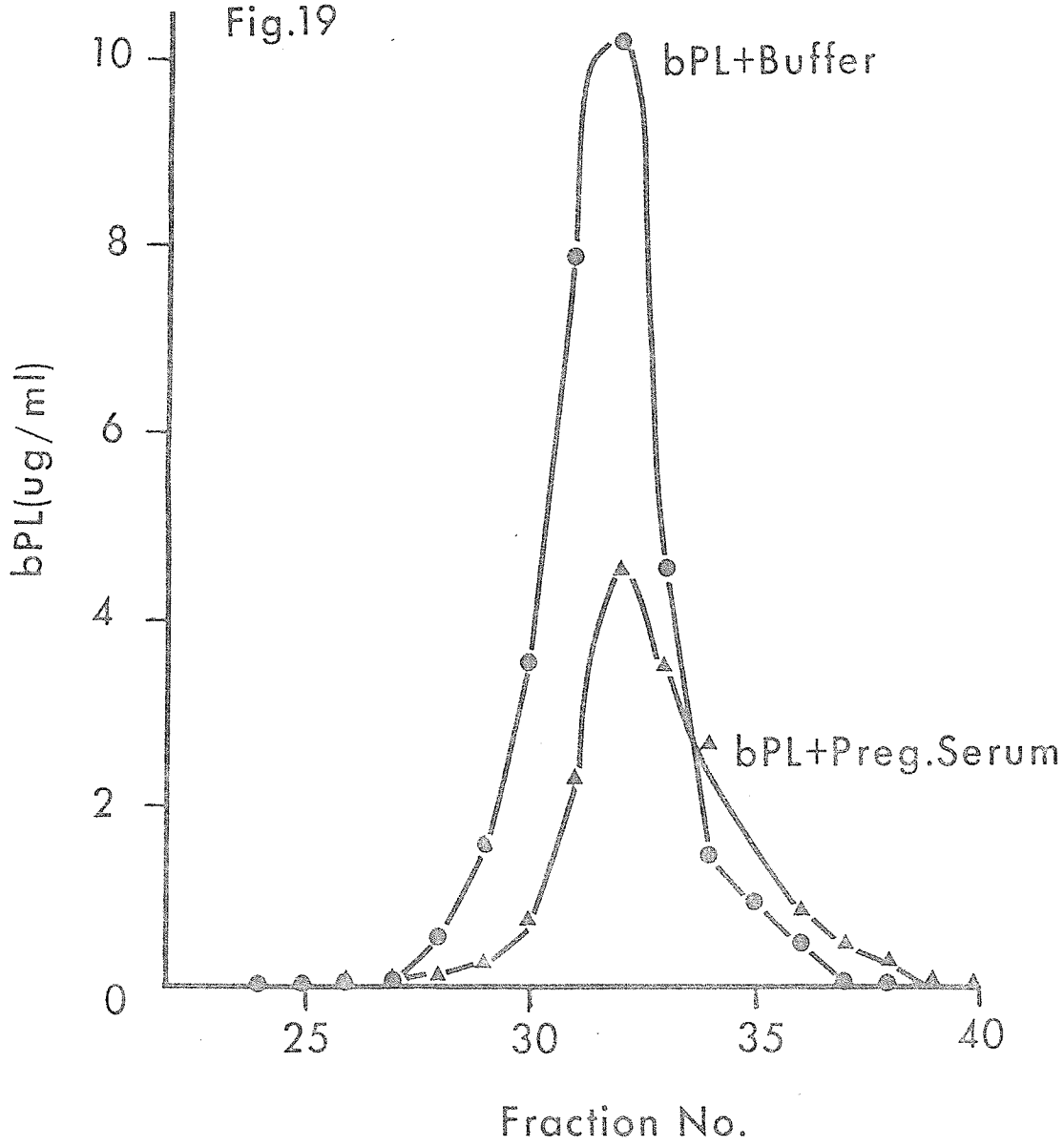

bPL+Buffer
bPL+Preg.Serum
bPL(ug/ml)
0
2
4
6
8
10
25
30
35
40
Fraction No.

Figure 20

Comparison of the distribution of immunoreactive bPL following isoelectric focusing of 50 ul aliquots of bPL (30 ng; △), bovine placental extract (45 ng bPL; ■), and pregnant cow serum (7.5 ng bPL; ●) in a polyacrylamide gel system of pH range 4-6.5. Following electrofocusing of the samples applied at the cathode, each lane of the gel was sliced into 21 segments and the individual slices eluted overnight at 4°C in 1 ml 1% BSA-PBS buffer, pH 7.4 containing 50 mM EDTA and 0.1% azide. An adjacent lane of the gel containing no sample was sliced in an identical manner and each segment eluted in 1 ml distilled water. Aliquots of the buffer in which elution of the sample gel slices occurred were assayed in the RIA-bPL . The left hand ordinate represents immunoassayable bPL. The pH (O) of the slices eluted in the distilled water was measured by pH electrode.

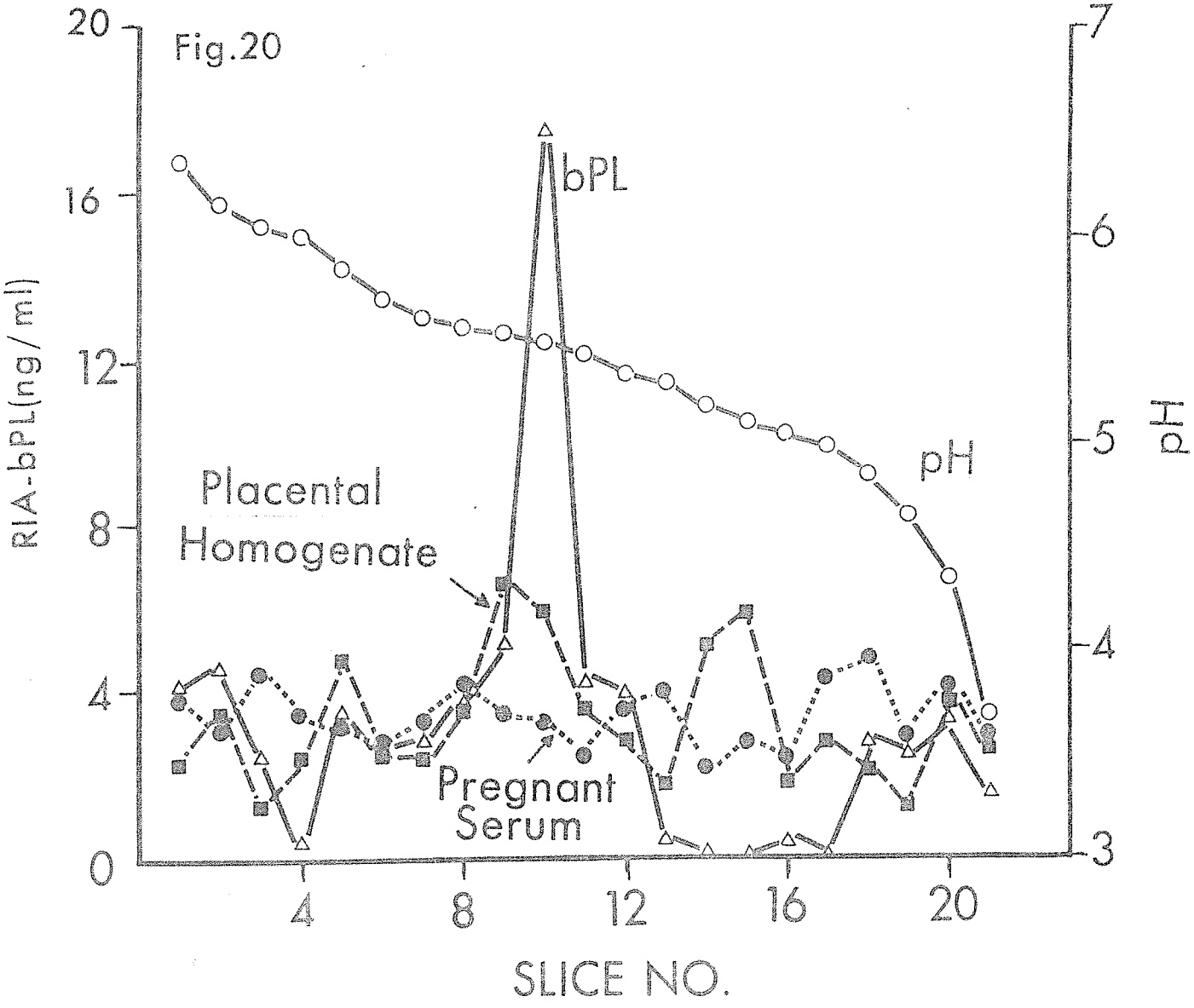


Fig.20
20
16
12
8
4
0
RIA-bPL(ng/ml)
bPL
pH
Placental
Homogenate
Pregnant
Serum
7
6
5
4
3
pH
4
8
12
16
20
SLICE NO.

RIA bPL is effected with the eluant from slice 10 (pH 5.5) of the pure bPL. Minor amounts of immunoreactive eluted material are seen at pH 6-5.9, pH 5.6-5.5, and pH 5.3-5.1 in the placental homogenate. Pregnant serum contains no eluted bPL-like immuno-activity in this system.

The search for a bPL-like component in bovine serum samples eluted from a Sephadex G-100 gel is shown in Figure 21. The 5 ml of eluted non-pregnant cow serum (chromatogram A) shows no apparent ability to bind to receptors (pooled, lyophilized eluates, A to G). A high molecular weight component(s) in pool B, however, exhibits the ability to displace ^{125}I-bPL in the RIA for bPL.

The immunoactive profile of pregnant cow serum (B) mimics that seen for non-pregnant serum. There appears to be displayed some receptor binding ability not attributable to a protein effect, pools F and G where bGH and bPRL would be expected to elute.

Chromatogram C depicts the G-100 activity profiles of pregnant cow serum (previously eluted and tested in B) to which was added pure bPL just prior to gel seiving. The predominant feature here is the coincident peaks of both receptor and immuno-activity seen in pool E where purified bPL would be expected to elute.

There was no detectable component in pregnant cow serum which exhibited either the immune or receptor binding characteristics of pure bPL.

Figure 21

Distribution of protein and bPL after gel chromatography of bovine serum samples. Proteins in each fraction were measured by absorbance at 280 nm (●). (The 2X50 cm Sephadex G-100 column was equilibrated, and eluted, with PBS buffer at a flow rate of 55 ml/hr. As indicated on the abscissa, 3 ml fractions were collected.) Fractions were then pooled according to the alphabetic designation in the figure, dialyzed against distilled water, lyophilized, and reconstituted with 1 ml normal saline. (Aliquots of these pools were then assayed in the somatotropic radioreceptor assay (RRA-hGH; ■) and in the RIA for bPL (▲).

A. Chromatography of 5 ml of non-pregnant cow serum.

B. Chromatography of 5 ml of pregnant cow serum.

C: Gel filtration of 4 ml of pregnant cow serum to which had been added 1 ml PBS buffer containing about 10 ug bPL.

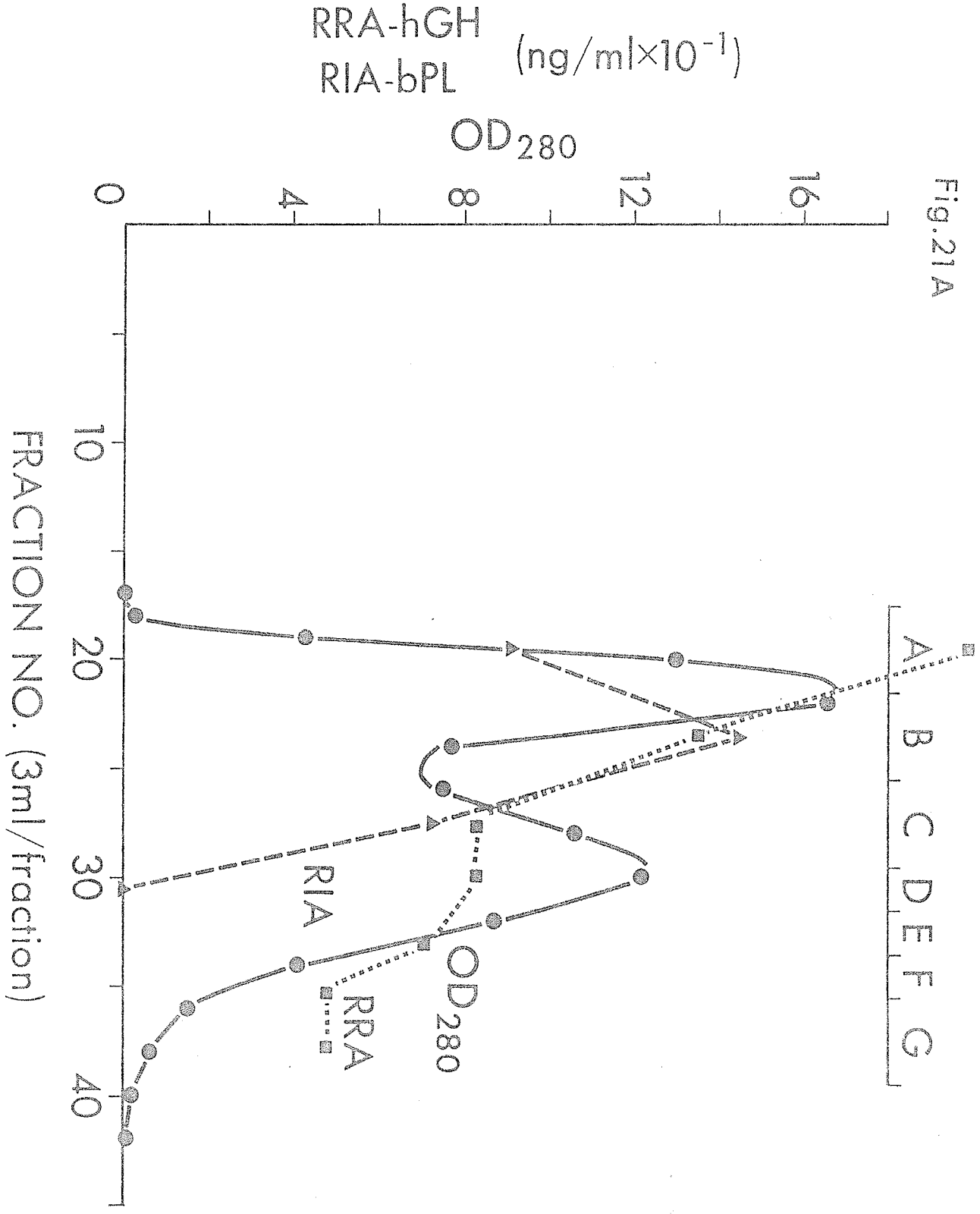
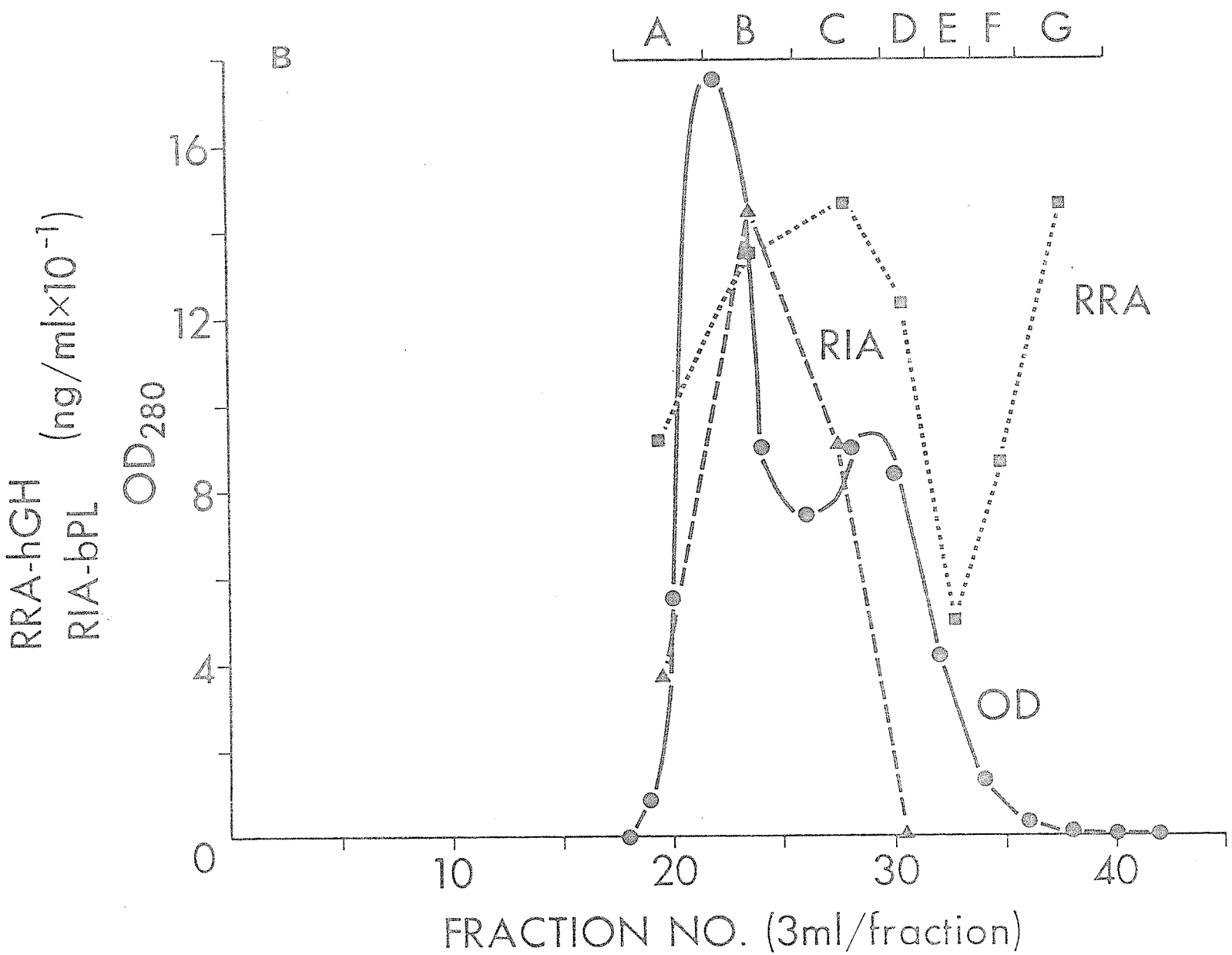
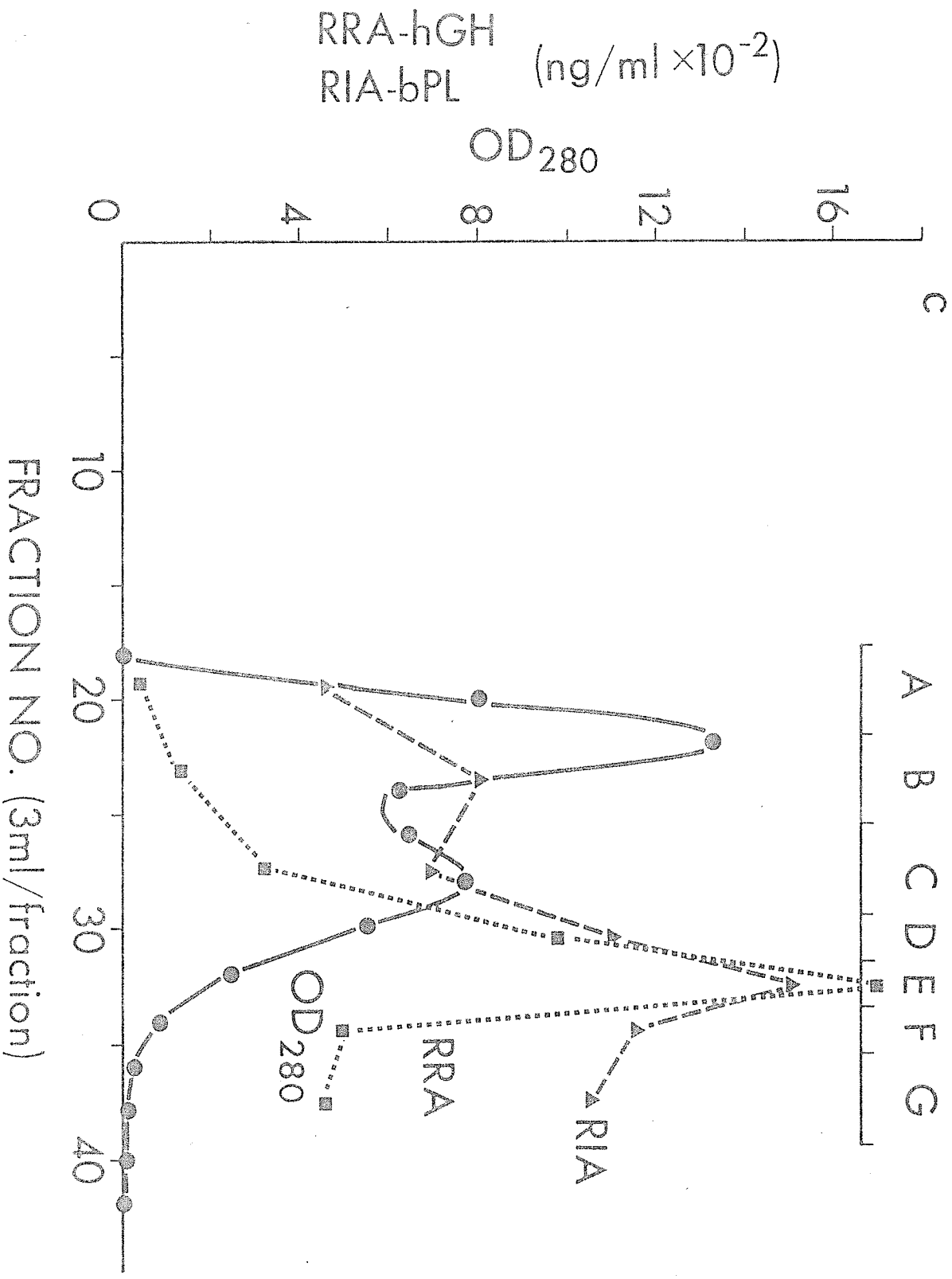

RRA-hGH
RIA-bPL
(ng/ml×10⁻¹)
OD280
0
4
8
12
16
10
20
30
40
FRACTION NO. (3ml/fraction)
RIA
RRA
OD280
A
B
C
D
E
F
G

Fig.21A


B
A B C D E F G
RRA-hGH
RIA-bPL
(ng/ml×10⁻¹)
OD280
16
12
8
4
0
RIA
RRA
OD
10
20
30
40
FRACTION NO. (3ml/fraction)


RRA-hGH
RIA-bPL
(ng/ml ×10⁻²)
OD280
0
4
8
12
16
A
B
C
D
E
F
G
FRACTION NO. (3ml/fraction)
10
20
30
40
OD280
RRA
RIA

H: BIOLOGICAL CHARACTERIZATION OF PURIFIED bPL

1) Displacement of ^{125}I-bPL from Rabbit Liver Membrane Receptors by hGH

The displacement and binding (inset) characteristics of ^{125}I-bPL and ^{125}I-hGH to pregnant rabbit liver membrane receptors are shown in Figure 22. The displacement curves of the two tracers are superimposable, indicating that the binding characteristics are virtually identical.

2) Displacement of ^{125}I-oPRL by bPL in the RRA for PRL

When purified bPL, standardized in the RRA-hGH, was assayed in the RRA-PRL it inhibited the binding of ^{125}I-oPRL in a parallel manner to oPRL and bPRL standards. (Figure 23) The bPL preparation, equipotent to hGH in competing for GH receptors, is shown here to be about 40% as potent as oPRL and approximately 50% as potent as bPRL.

3) Aspects of bPL Binding to and Displacement from Lactogenic and Somatotropic Receptor Sites

Figure 24 shows the SDS polyacrylamide gel radioautograph of ^{125}I-bPL before absorption to and after removal from pregnant rat liver membranes. Partially purified bPL was used as tracer in this study to determine whether more than one component of placental origin possessed the ability to bind to the lactogenic receptor sites in rat liver. Lane 4 of the photograph displays the radioactive profile of the tracer that was incubated with the receptor. Lane 1 shows the single

Figure 22

Competative displacement of ^{125}I-hGH (●) and ^{125}I-bPL (△) from rabbit liver membrane receptors by hGH. The inset shows the specific binding observed for both systems. The concentration of the membrane protein used for both systems was 80 ug/ml.

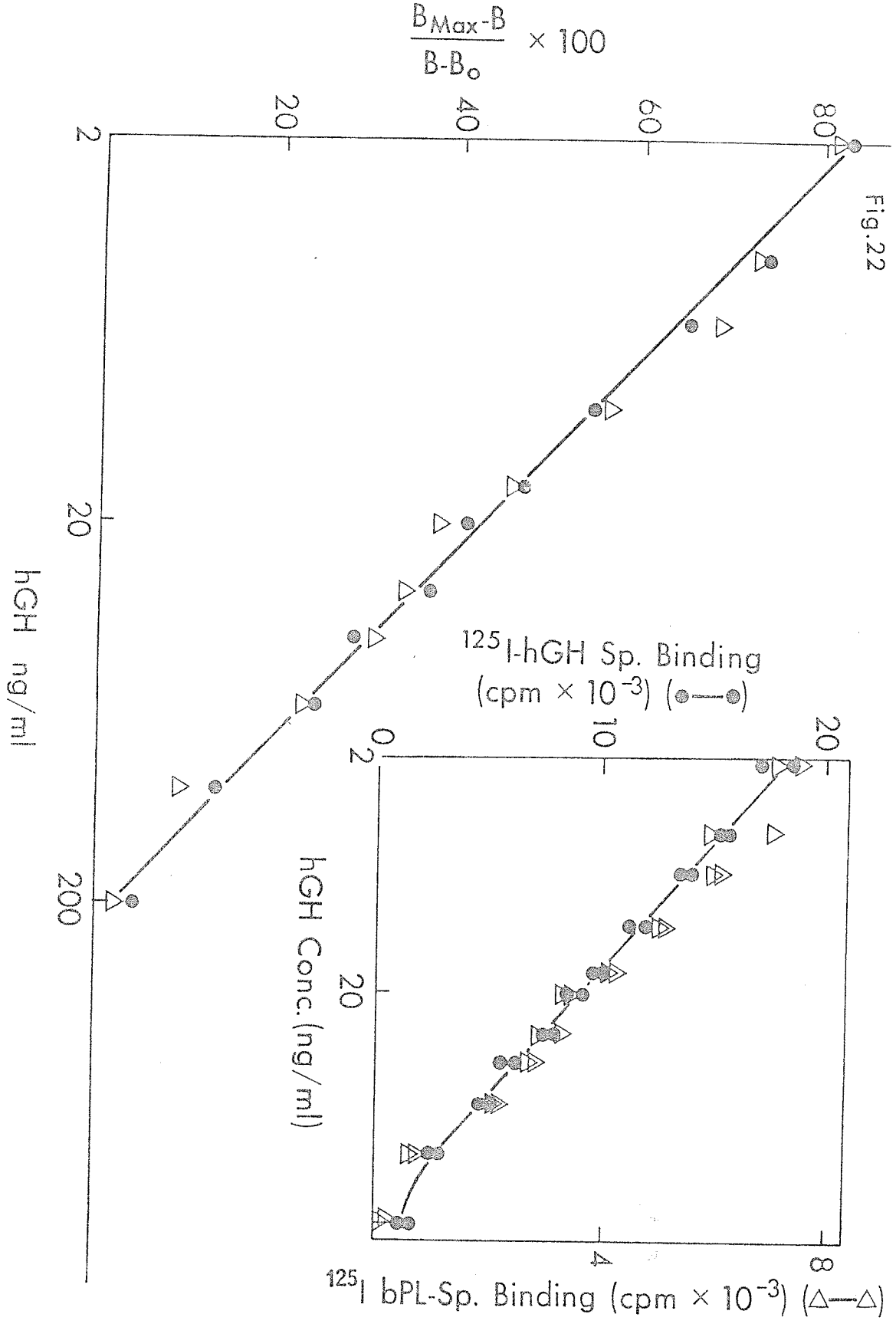

$\frac{B_{Max}-B}{B-B_o} \times 100$
2
20
40
60
80
2
20
200
hGH ng/ml
^{125}I-hGH Sp. Binding
(cpm × 10^{-3}) (●—●)
0
10
20
2
20
hGH Conc. (ng/ml)
4
8
^{125}I bPL-Sp. Binding (cpm × 10^{-3}) (△—△)

Fig. 22

Figure 23

Displacement of ^{125}I-oPRL by oPRL, bPRL, and bPL in the lactogenic radioreceptor assay (RRA-oPRL) using late pregnant rat liver as the source of receptor. Crude rat liver membranes were incubated with ^{125}I-oPRL in the presence of displacing hormone. Increasing the concentration of displacing hormone decreases the amount of ^{125}I-oPRL bound. The ordinate represents ^{125}I-oPRL bound to prolactin binding sites while the abscissa represents the concentration of hormone added to each assay tube.

Fig.23

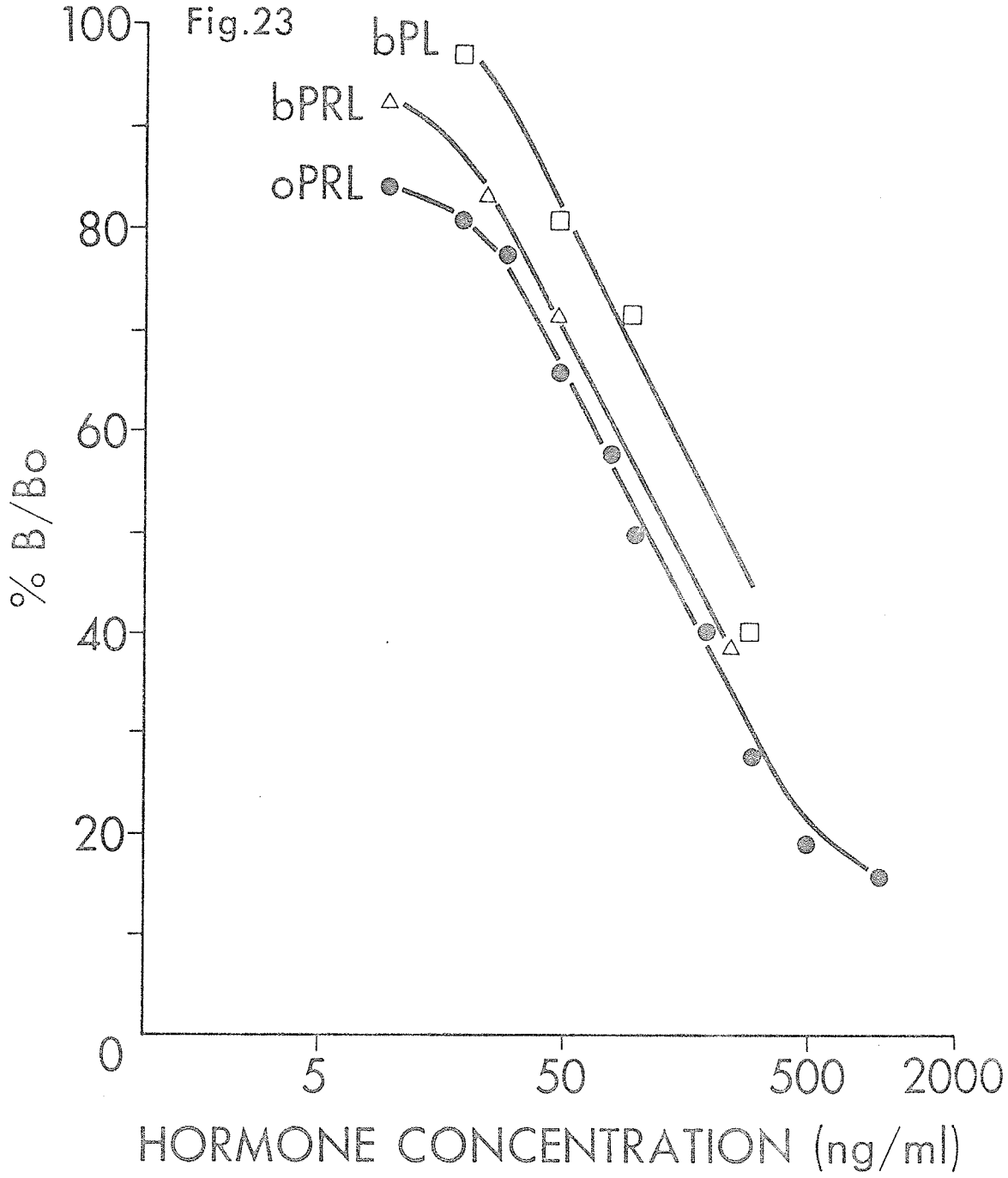
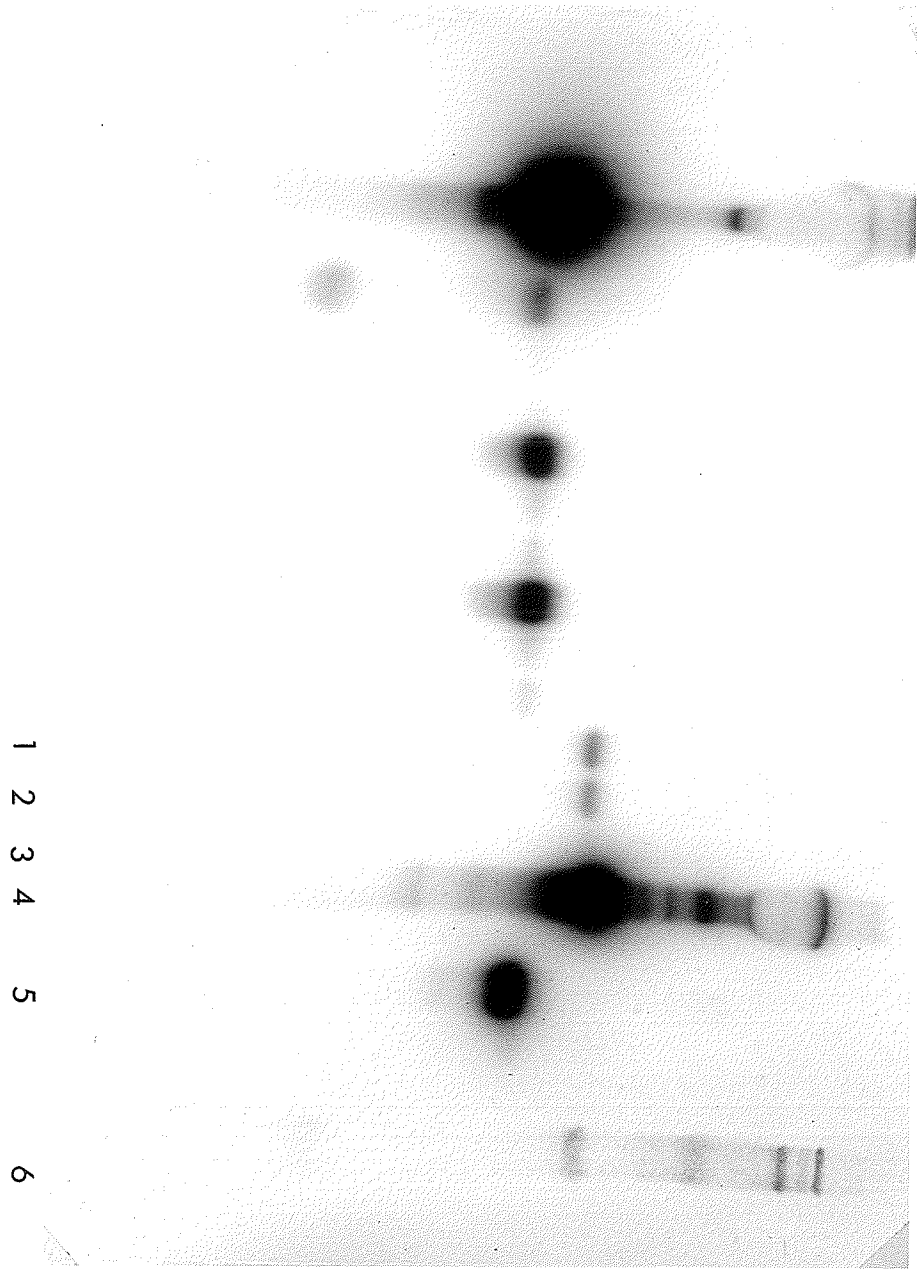

bPL
bPRL
oPRL
100
80
60
40
20
0
% B/Bo
5
50
500
2000
HORMONE CONCENTRATION (ng/ml)

Figure 24

Sodium dodecyl sulfate polyacrylamide gel radioautograph of ^{125}I-bPL stripped from lactogenic receptor sites. ^{125}I-bPL was incubated with excess pregnant rat liver membranes in the presence and absence of excess hGH or oPRL. Membranes were subsequently washed of unbound tracer and absorbed. ^{125}I-bPL was then stripped from the membranes with 5 M $MgCl_2$. Membranes were removed by centrifugation and the supernatant was dialyzed against distilled water to remove the $MgCl_2$. The dialyzed supernatant was applied to the SDS gel.

lane 1	^{125}I-bPL bound by and stripped from lactogenic membranes
lane 2	dialyzed supernatant from incubation mixture of ^{125}I-bPL, excess oPRL, and membranes
lane 3	dialyzed supernatant from incubabation mixture of ^{125}I-bPL, excess hGH and membranes
lane 4	^{125}I-bPL
lane 5	^{125}I-bPRL bound by the lactogenic membranes
lane 6	standards (as for Figure 10)

FIGURE 24



radiolabelled component of the tracer, at a molecular weight of about 35,000, that was initially absorbed to, and later eluted from, the receptors and then applied to the gel. This 35,000 molecular weight component is bound specifically to membrane receptors. Lane 3, which shows no radiolabelled bands. represents the control of ^{125}I-bPL plus receptor in the presence of excess hGH. The human hormone has specifically prevented binding of labelled bPL to the receptor. Lane 2 demonstrates a slight reduction in radioactive intensity of the bound and stripped bPL caused by the incubation of the tracer and receptor in the presence of excess oPRL. Lane 5 represents ^{125}I-bPRL that was stripped from the rat liver lactogenic receptor by excess oPRL.

Table 5 compares maximum ability of several lactogenic and somatotropic hormones to displace heterologous, iodinated hormones from either lactogenic (rat liver) or somatotropic (rabbit liver) membrane receptors. hGH and bPL, when in excess, demonstrate great similarity in their ability to displace virtually 100% of all the tracers tested from both receptor systems.

Table 6 demonstrates a further similarity in the biological activities of bPL and hGH by comparing the ability of the two hormones to modulate the specific hGH receptors present on IM-9 lymphocytes. bPL, like hGH, is able to reduce the % specific binding of ^{125}I-hGH to the human cells in the assay system.

TABLE 5

DISPLACEMENT OF VARIOUS HORMONE TRACERS FROM LACTOGENIC AND SOMATOTROPIC RECEPTORS BY EXCESS HETEROLOGOUS HORMONES

TRACER[1]	DISPLACING HORMONE[1] (2 ug/ml)	% SPECIFIC BINDING[2] RAT LIVER	% SPECIFIC BINDING[2] RABBIT LIVER	% DISPLACEMENT RELATIVE TO DISPLACEMENT BY EXCESS HOMOLOGOUS HORMONE[2] RAT LIVER	% DISPLACEMENT RELATIVE TO DISPLACEMENT BY EXCESS HOMOLOGOUS HORMONE[2] RABBIT LIVER
^{125}I-bPL	bPL	8.2	18.4		
	hGH			92	91
	bGH			23	79
	bPRL			78	76
	oPRL			48	
^{125}I-bGH	bGH	4.0	10.2		
	hGH			77	94
	bPL			101	108
	bPRL			21	93
	oPRL			14	
^{125}I-bPRL	bPRL	31.1	6.9		
	hGH			101	111
	bGH			18	75
	bPL			98	106
	oPRL			95	
^{125}I-oPRL	oPRL	28.8			
	hGH			114	
	bGH			18	
	bPRL			113	
	bPL			106	
^{125}I-hGH	hGH		19.0		
	bPL				100
	bGH				76
	bPRL				78

[1]WHERE: b = BOVINE, h = HUMAN, o = OVINE; PL = PLACENTAL LACTOGEN, GH = GROWTH HORMONE, PRL = PROLACTIN

[2]BLANK SPACES INDICATE UNTESTED CONDITION

TABLE 6

Effect of hGH and bPL on the modulation of hGH receptor Concentrations of IM-9 Lymphocytes

Preincubation Condition	Assay Conditions	% Specific Binding
buffer (modified Hepes)	^{125}I-hGH	3.8
buffer	^{125}I-hGH + hGH (1 ug/ml)	-0.9
buffer + hGH (1 ug/ml)	^{125}I-hGH	-1.1
buffer + hGH (1 ug/ml)	^{125}I-hGH + hGH (1 ug/ml)	-1.0
buffer + bPL (2 ug/ml)	^{125}I-hGH	0.4
buffer + bPL (2 ug/ml)	^{125}I-hGH + hGH (1 ug/ml)	-1.2

I: in vitro BIOASSAY OF bPL

1) Characterization of bPL in the Nb2 Bioassay

Figure 25 illustrates the typical standard curve generated by either bPL or bPRL in the Nb2 lactogenic bioassay.

The effect of addition of antibodies to either bPL or bPRL to Nb2 culture dishes in the presence of either lactogen (bPL or bPRL) is shown in Figure 26. Addition of the homologous antiserum abolishes all cell stimulating activity while the heterlogous antiserum has no effect on cell division caused by the hormone.

Figure 27 A demonstrates the increased effect on cell division by the addition of pregnant cow serum to bPRL standards. Addition of excess homologous antiserum (a-bPRL) blocks all endogenous and exogenous cell proliferating activity in the serum and standard bPRL dishes (3).

Figure 27 B shows the results of a repetition of the events in A with the exception that bPL replaced bPRL and a-bPL replaced a-bPRL. The antiserum to bPL was unable to diminish the endogenous cell stimulating effect of pregnant cow serum.

Table 7 shows the distribution of bPL following the electrofocusing of a partially purified preparation of bPL in the Sephadex IEF system. Aliquots of the eluates from lanes 14, 15, 16, 19, and 20 were labelled 1-5 respectively and focused in a polyacrylamide gel IEF system. The staining pattern of the 5 eluates following

Figure 25

Stimulation of the growth of stationary Nb2 lymphoma cell cultures by bPRL (●) and bPL (▲). Concentraion of bPRL standards was determined on a weight basis while bPL standard concentrations are hGH equivalents of pure bPL based on values obtained in the RRA for hGH.

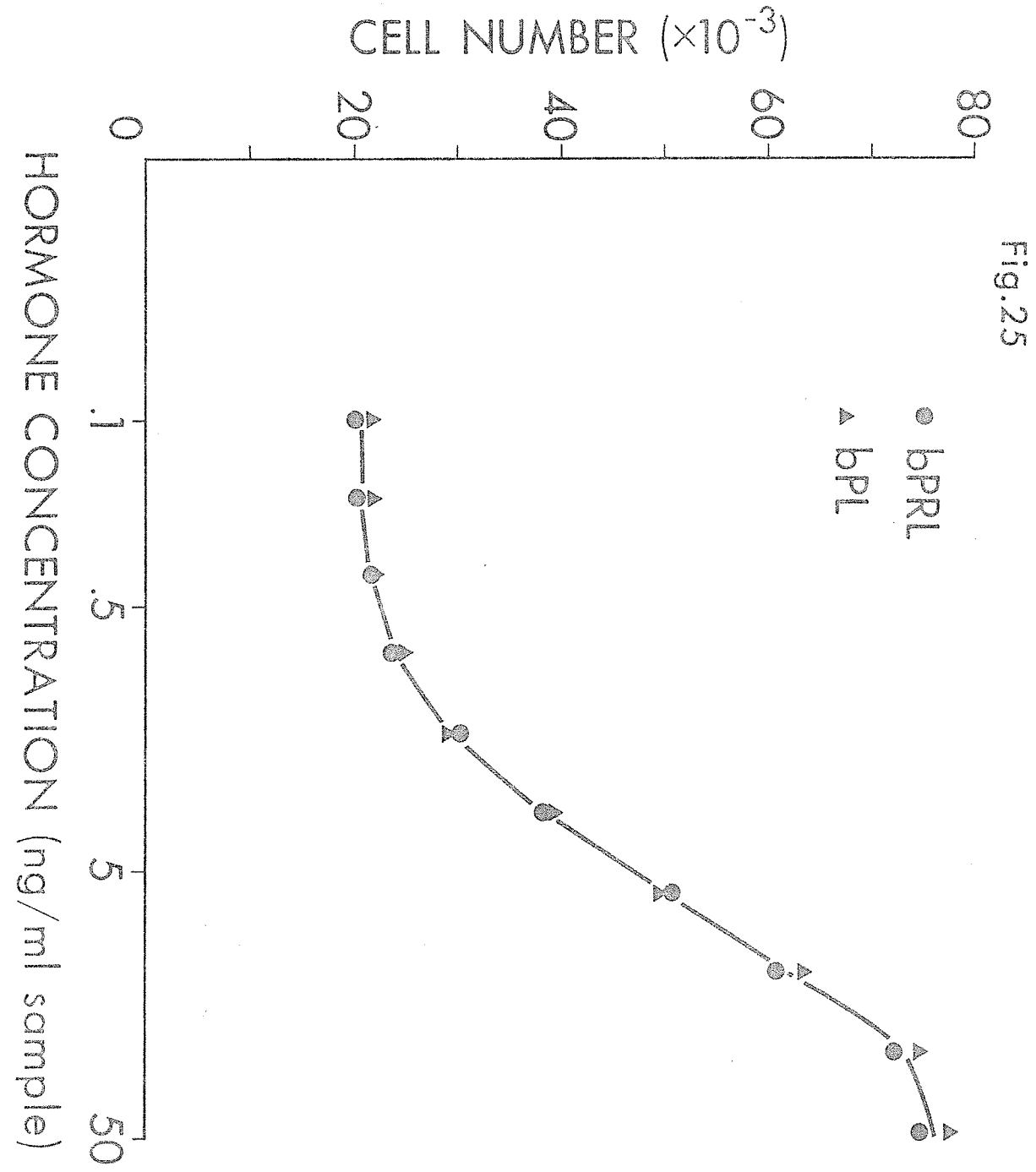

CELL NUMBER (×10⁻³)
0
20
40
60
80
HORMONE CONCENTRATION (ng/ml sample)
.1
.5
5
50
bPRL
bPL

Fig.25

Figure 26

Effect of bPRL and bPL, in the presence and absence of antibody to bPRL (a-bPRL) and antibody to bPL (a-bPL), upon proliferation of Nb2 lymphoma cell cultures. The ordinate indicates the number of cells in 1 ml of a 1:5 dilution of each culture dish. The abscissa represents the concentration of the three standard hormone solutions from which a 50 ul aliquot was drawn and added to the cells in culture. a-bPRL (1:100) and a-bPL (1:10), when used, were added in 50 ul aliquots to each culture dish just prior to the addition of the bovine hormones. Final concentrations of the antisera in the culture dishes (2 ml) were 1:4000 for a-bPRL and 1:400 for a-bPL.

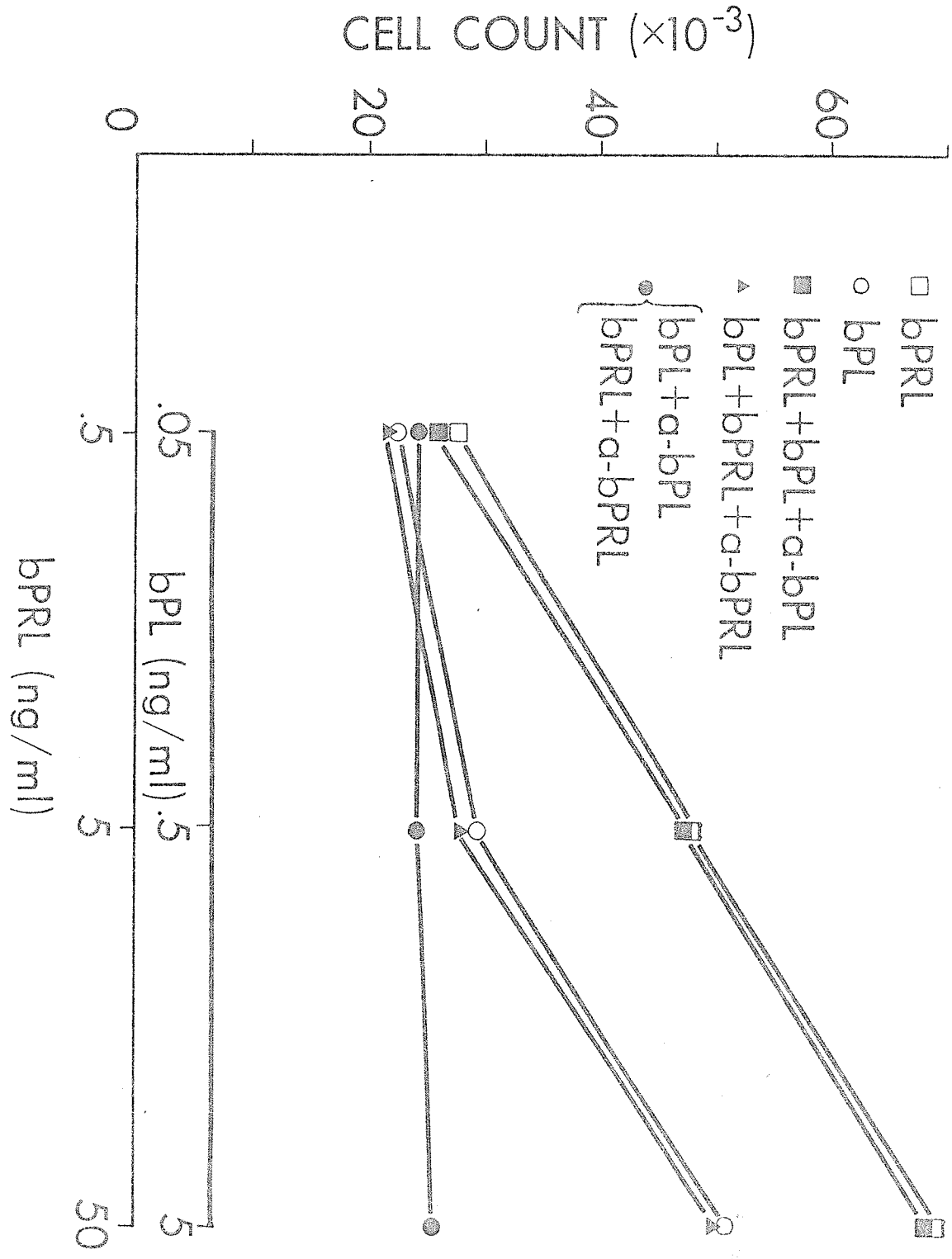

CELL COUNT (×10⁻³)
0
20
40
60
□ bPRL
○ bPL
■ bPRL+bPL+a-bPL
▲ bPL+bPRL+a-bPRL
bPL+a-bPL
bPRL+a-bPRL
bPRL (ng/ml)
.5
5
50
bPL (ng/ml) .5
.05
5

Fig. 26

Figure 27

A: The effect of the addition of pregnant cow serum (S) and/or i)normal rabbit serum (NRS at 1:100), ii) antiserum against bPRL (a-bPRL at 1:100) upon the proliferation of Nb2 lymphoma cells in the presence of bPRL.

1) bPRL; bPRL + NRS

2) bPRL + S; bPRL + S + NRS

3) bPRL + S + a-bPRL

B: The effect of the addition of pregnant cow serum (S) and/or i)normal rabbit serum (NRS at 1:10), ii) antiserum against bPL (a-bPL at 1:10) upon the proliferation of Nb2 lymphoma cells in the presence of bPL

1) bPL; bPL + NRS

2) bPL + S; bPL + S + NRS

3) bPL + S + a-bPL

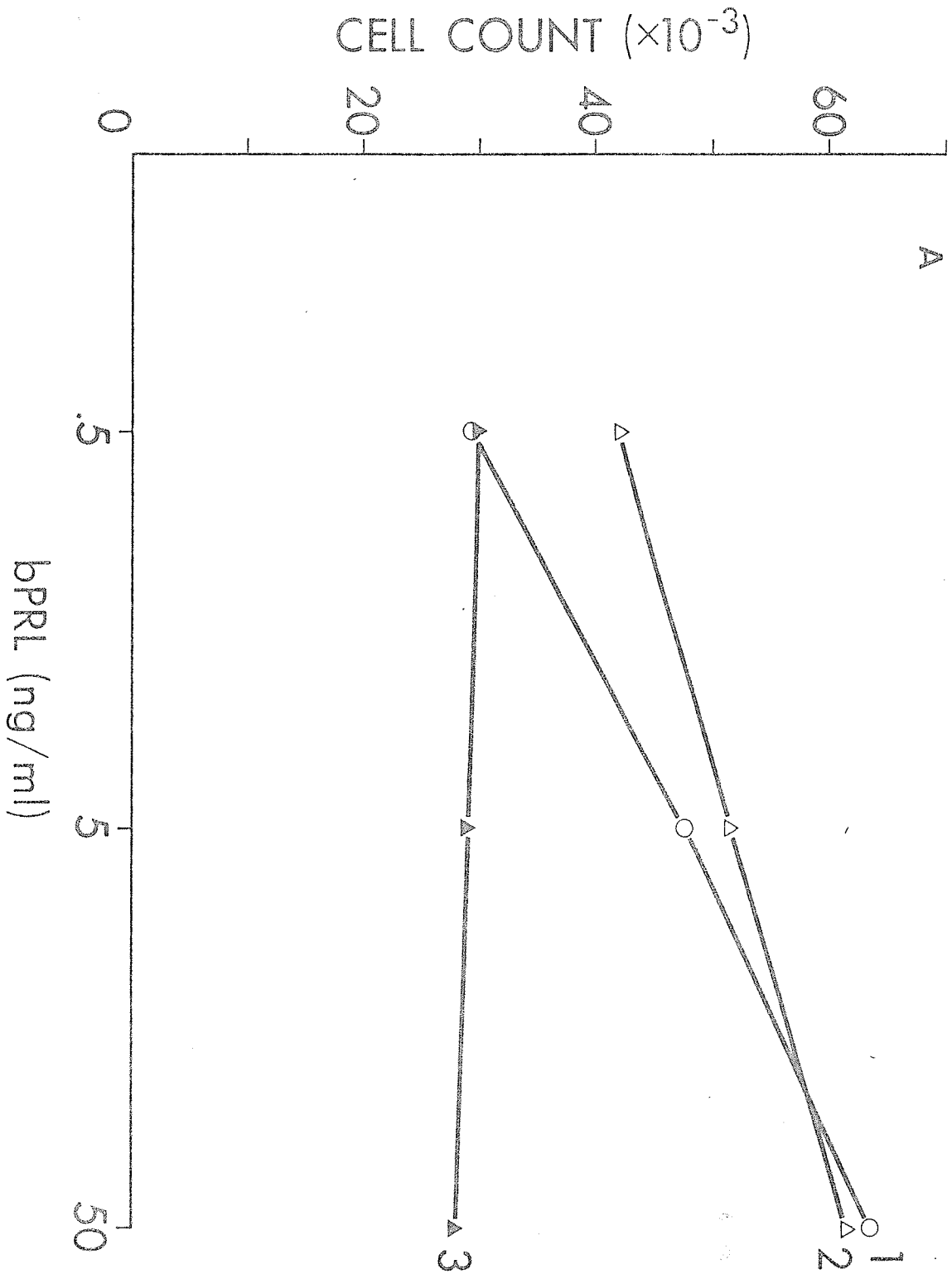


Fig. 27
A

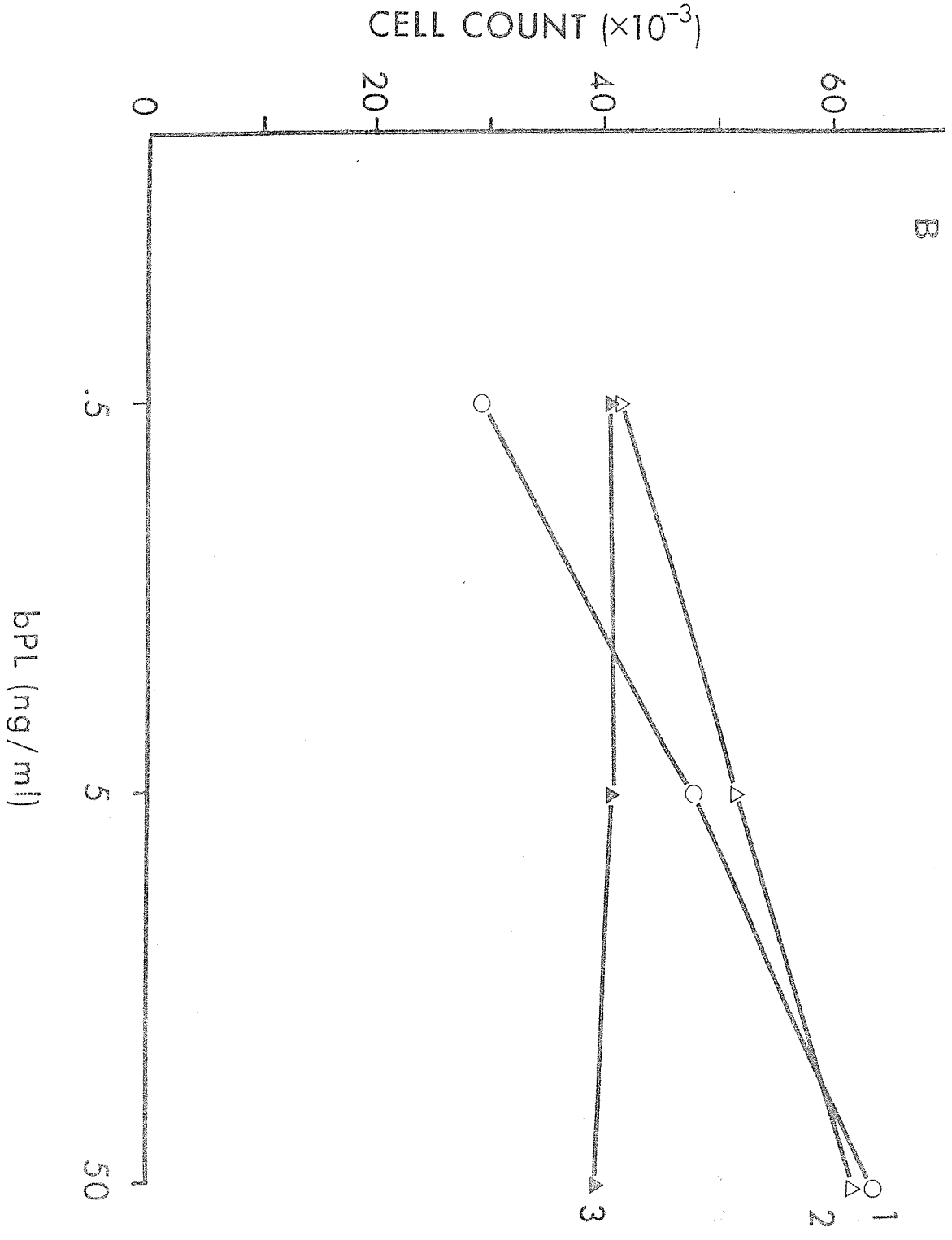

CELL COUNT (×10⁻³)
0
20
40
60
B
.5
5
50
bPL (ng/ml)
1
2
3

Table 7

bPL ELUTED FROM SEPARATE LANES OF SEPHADEX GEL FOLLOWING ISOELECTRIC FOCUSING

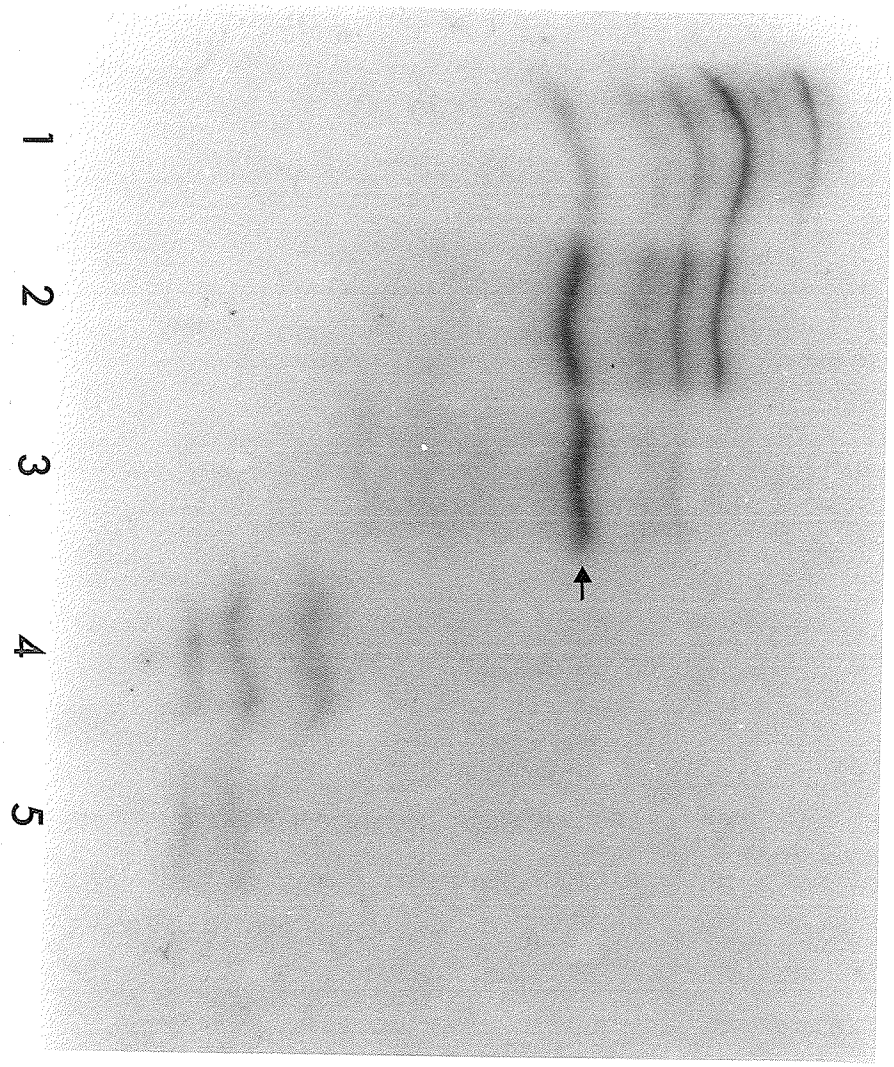
Lane No.	bPL (ug/ml)[1]	pH
1-13	0	3.1-5.2
14	17	5.3
15	63	5.4
16	74	5.6
17	49	5.7
18	18	5.8
19	7	6.0
20	3	6.1
21-30	0	6.3-8.1

[1] bPL concentration measured by RRA-hGH

Figure 28

Protein staining pattern of partially purified bPL following polyacrylamide gel (PAG) isoelectric focusing (IEF) across a pH range of 4-6.5. Impure bPL was initially electrofocused in a Sephadex-IEF system. The gel was divided into 30 lanes and the gel lanes (14-20) between pH 5.3-6.1 (the portion of the gel to which bPL activity had been localized; see Table 7) were eluted with distilled water. An aliquot of the eluate from lanes 14, 15, 16, 19, and 20 was electrofocused in duplicate in the PAGIEF system (the applied eluates were labelled 1-5 respectively). An aliquot of each eluate was refocused in duplicate in the PAG IEF system. Following focuaing the gel was cut into halves with each half containing one focused lane of each applied aliquot. One half of the gel was then stained for protein and the other half had lane 3 sliced into 20 segments with each slice eluted in 1 ml 0.1% BSA-PBS buffer. Lane 3 of the PAG was chosen to be segmented and assayed because it represented the lane from the Sephadex IEF gel which had the highest concentration of bPL (lane 16). Aliquots of the buffer were then assayed in the RRA for oPRL, RRA for hGH, RIA for bPL, and Nb2 lymphoma cell bioassay. (See Table 8 for results of assays). Maximum bPL activity as measured by bio- and receptor assay corresponding to eluted slice no. 13 at pH 5.5, is indicated by the arrow.

FIGURE 28



electrofocusing across a pH range of 4-6.5 is shown in Figure 28. Table 8 presents the receptor, bio-, and immuno-activity present in eluates of the 20 slices of the polyacrylamide gel lane number 3. A good correlation is seen for bPL levels obtained in the two receptor assays and in the Nb2 bioassay. Predominant activity is found at pH 5.5 while eluates with minor activity are detected at pH 5.8 and 5.3. The absence of any bPRL and bGH was confirmed by subjecting the eluates to RIA for either bPRL or bGH (not shown). The RIA for bPL presents spurious results for the eluted gel slices.

Figure 29 displays the standard curve and sensitivity in the homologous RIA for bPRL. No cross reaction at 1 ug/ml was seen with either bPL or bGH (not shown).

Figure 30 displays the characteristic standard curve and sensitivity of the heterologous RIA for bGH. bPL and bPRL at concentrations of 1 ug/ml did not cross-react in the assay.

Figure 31 presents the results obtained when acrylamide gel electrofocused ^{125}I-bPL was studied in the RRA for hGH, RIA for bPL, and Nb2 bioassay. The major receptor active tracer was eluted from the gel at pH 5.5 while bioactive tracer appeared at pH 5.5 and 5.3. Maximum tracer precipitation by anti-bPL antiserum occurred with the pH 5.5 eluate, however a second major component of the tracer at pH 6 was also precipitated. Displacement of the various eluted tracer constituents by pregnant cow serum is maximal for the eluate at about

TABLE 8

DISTRIBUTION OF RECEPTOR, BIO-, AND IMMUNOACTIVITY FOLLOWING ISOELECTRIC FOCUSING OF bPL IN POLY-ACRYLAMIDE GEL

SLICE NO.	pH	RRA-hGH	RRA-oPL	Nb2	RIA-bPL
1	6.6				215
2	6.4				165
3	6.3				30
4	6.2				
5	6.1				
6	6.0				
7	5.9			5.2	28
8	5.8	22	10	22.8	
9	5.7			7.2	
10	5.7			5.2	
11	5.7			6.8	
12	5.6	22	24	29.6	25
13	5.5	290	280	312	115
14	5.4	205	205	220	
15	5.4			6	
16	5.3			6.4	
17	5.1				
18	5.0				25
19	4.6				73
20	4.1				

[1]ELUTED ACTIVITY WAS DETERMINED FOR ALL SLICES IN EACH ASSAY; BLANK SPACES = NO MEASUREABLE ACTIVITY.

Figure 29

The standard curve of the homologous radioimmunoassay for bPRL. The lower sensitivity of the assay is 2 ng/ml. Purified bGH and bPL show no cross-reaction in this assay at concentrations as high as 1000 ng/ml (not shown). Bovine placental extracts do not cross-react at hGH-equivalent activity (RRA-hGH) of 2000 ng/ml (not shown).

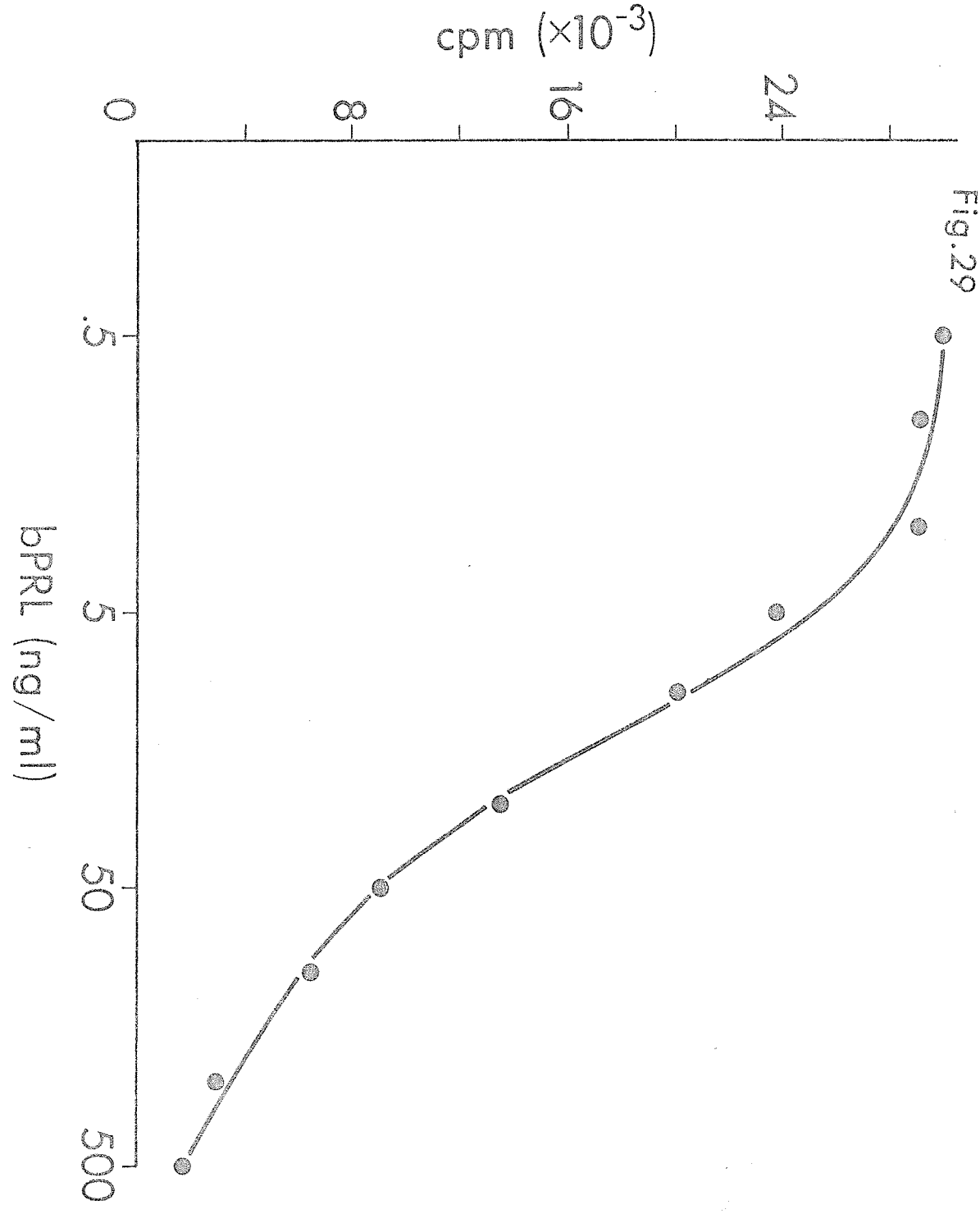


Fig. 26

Figure 30

The standard curve of the heterologous radioimmunoassay for bGH. Standards were oGH and the tracer was ^{125}I-bGH. The first antibody used in this assay was generated in rabbits against oGH. Purified bPRL and bPL show no cross-reaction in the assay at 1000 ng/ml (not shown). Bovine placental extracts at hGH-equivalent concentrations of 2000 ng/ml, as determined in the RRA-hGH, show no cross-reaction (not shown).

Fig.30

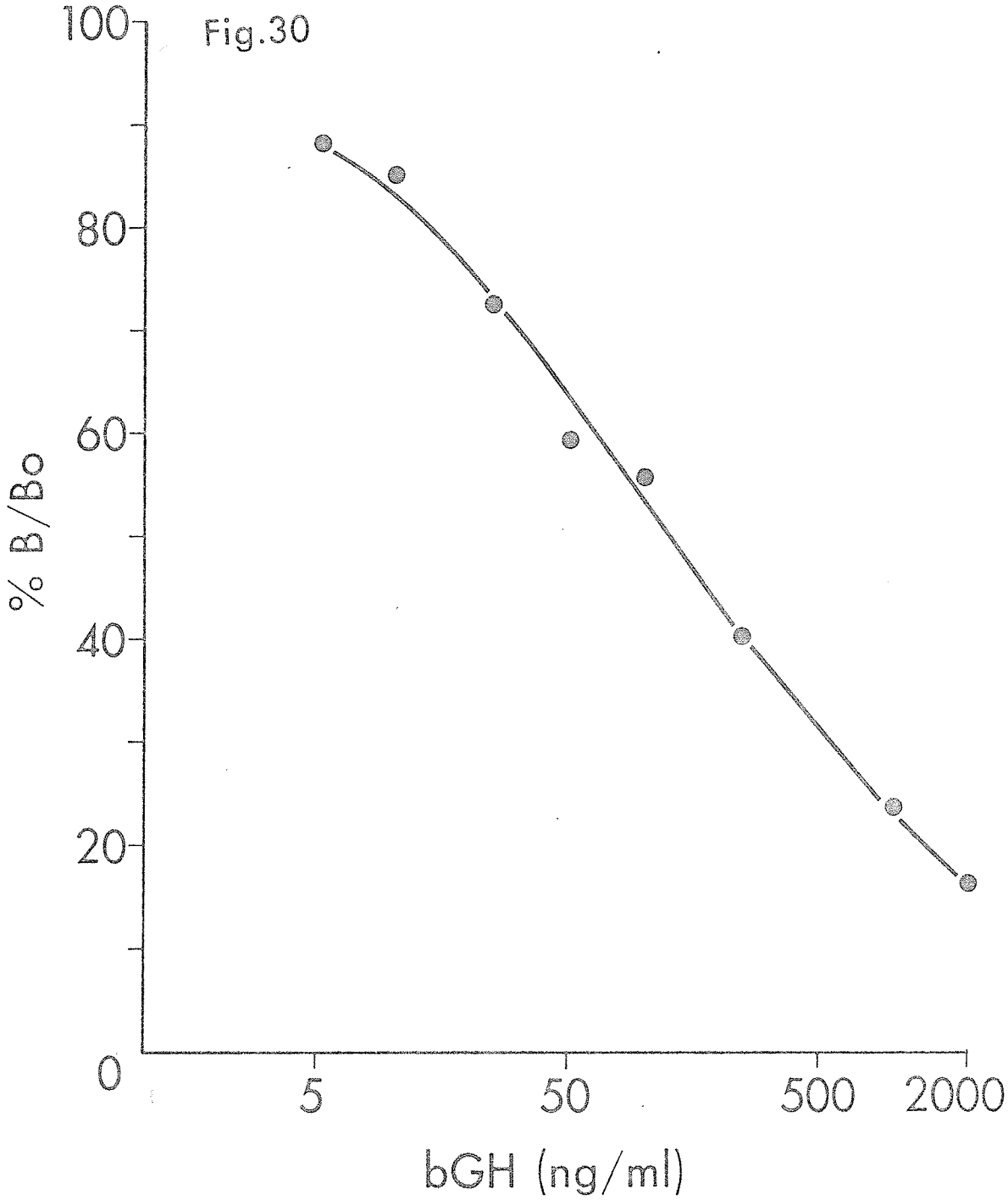


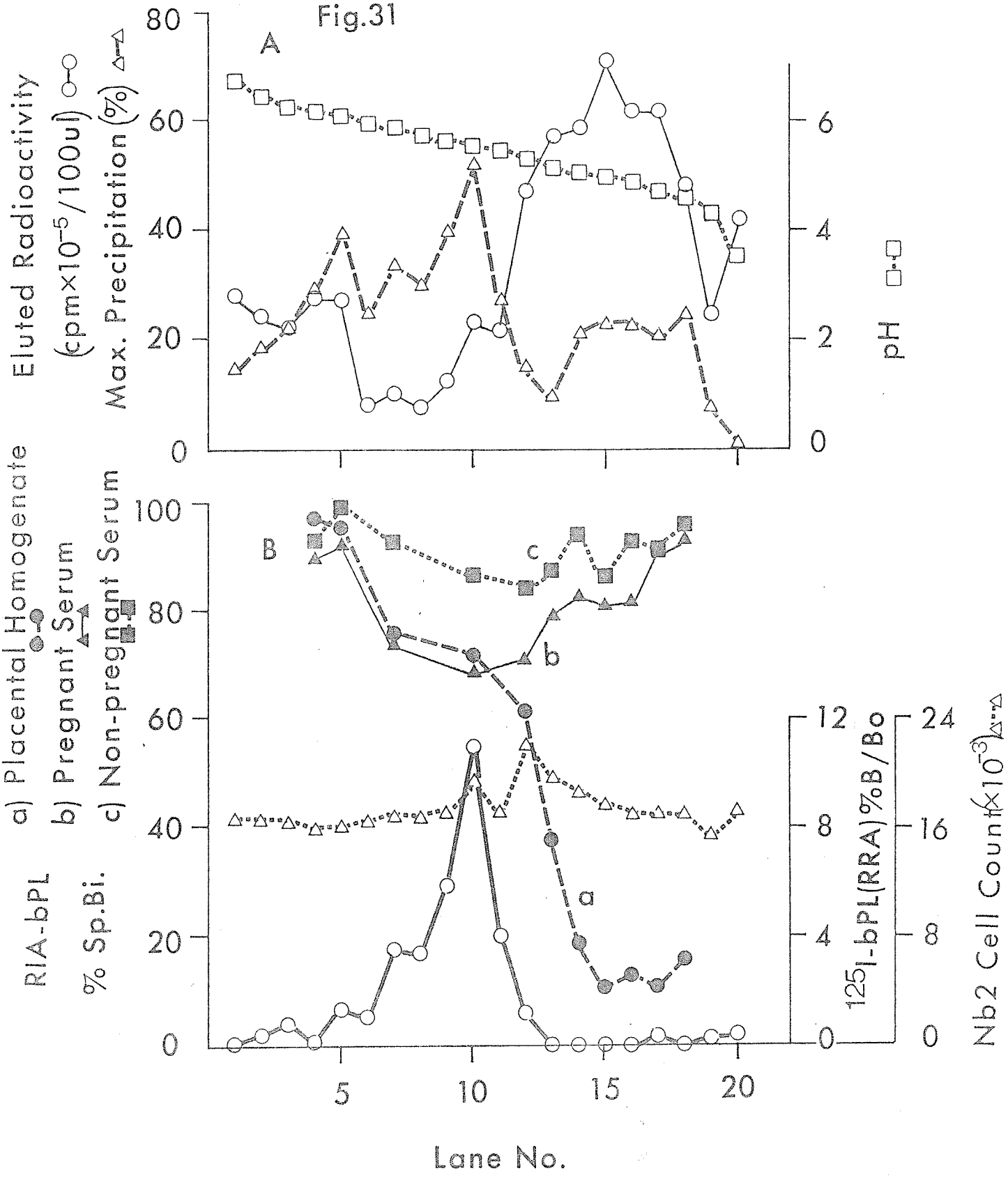
Figure 31

The radioactive distribution and the receptor, immuno- and bioactivity of ^{125}I-bPL following isoelectric focusing in a polyacrylamide gel (PAG) system of pH range 4-6.5. Focusing of ^{125}I-bPL in the PAG system was followed by slicing the radioactive lane into 20 segments and eluting these slices in 1 ml of 1% BSA-PBS buffer overnight at 4^{o}C. An adjacent lane was sliced identically and eluted in 1 ml distilled water.

A. Aliquots of the PBS eluates were screened in a gamma counter (O) to determine the radioactive profile of focused ^{125}I-bPL. Tracer eluted from each slice was precipitated in the presence of excess antibodies to bPL (△). The pH profile (□) at completion of focusing was determined by measuring the pH of the distilled water eluates.

B. Aliquots of ^{125}I-bPL eluted from each gel slice were tested for i) ability to bind specifically to growth hormone receptors on rabbit liver (O), ii) ability to stimulate proliferation of Nb2 lymphoma cells in culture (△), and iii) competetive displacement from antibodies to bPL by bovine placental extract (●), pregnant cow serum (▲), and non-pregnant cow serum (■).

Fig.31


A
B
Eluted Radioactivity (cpm×10⁻⁵/100ul)
Max. Precipitation (%)
pH
RIA-bPL
% Sp.Bi.
a) Placental Homogenate
b) Pregnant Serum
c) Non-pregnant Serum
125I-bPL(RRA) %B/Bo
Nb2 Cell Count(×10⁻³)
Lane No.

pH 5.5. Non-pregnant serum displaces about 10% of all eluted radioactivity. The placental homogenate causes maximal displacement with the tracer eluted from gel slices at pH 5.5 to 5.0.

2) Measurement of bPL in Bovine Fluids

Table 9 presents the values of lactogenic activity (all attributable to bPRL) present in bovine milk samples from 70-206 days of pregnancy. No bPL is found by bioassay.

Table 10 lists lactogenic activity attributable to both bPRL and bPL in bovine serum and amniotic fluid samples. Two pregnant serum samples show minimal levels of bPL by bioassay. The uterine vein and fetal serum samples, however, demonstrate significant levels of bPL. Amniotic fluid appears to contain a real lactogenic component attributable to bPL.

Figure 32 presents the distribution of lactogenic bioactivity in commercial fetal calf serum (FCS) following electrofocusing in the Sephadex gel system. Most of the lactogenic activity is attributable to bPL with the majority of the bPL focusing at pH 5.5-5.6 in the gel.

TABLE 9

IMMUNOREACTIVE bPRL AND LACTOGENIC BIOACTIVITY IN MILK OF THE PREGNANT AND NON-PREGNANT COW[1]

COW	DAY OF PREGNANCY	RIA-bPRL[2]	TOTAL	Nb2[3] bPRL	bPL
S. MYRA	206	4	4	4	0
R. NETTIE	168	7	9	10	0
M. QUEEN	138	10	15	13	0
B. LEORA	101	3	2	3	0
T. NORA	70	6	5	5	0
JOANNE	NON-PREG.	7	6	7	0
JOANNA	NON-PREG.	6	6	6	0

[1] ALL VALUES IN NG/ML

[2] SKIM MILK

[3] WHOLE MILK

TABLE 10

IMMUNOREACTIVE bPRL AND BIOACTIVE bPRL AND bPL IN BOVINE SERUM AND AMNIOTIC FLUID SAMPLES[1]

	SPECIMEN	DAY OF PREGNANCY	RIA-bPRL	TOTAL	Nb2 bPRL	bPL
STEER SERUM	1		24	21	18	0
	2		17	39	33	0
	3		12	13	10	0
COW SERUM	1	NON-PREG.	28	35	32	0
	2	NON-PREG.	70	53	54	0
	3	NON-PREG.	27	32	31	0
	4	60[2]	30	48	46	0
	5	135[2]	82	94	93	0
	6	270	16	21	19	0
	7	269	5	5	5	0
	8	251	21	35	34	0
	9	126	8	14	14	3
	10	101[2]	21	32	30	3
UTERINE VEIN SERUM	1	120[2]	45	115	62	27
	2	200[2]	85	450	450	11
	3	200[2]	80	150	110	7
FETAL SERUM	1	180	*	80	80	3
	2	180[2]	*	31	4	29
	3	120[2]	9	25	11	14
	4	200[2]	70	*	77	20
	5	200[2]	40	87	45	19
AMNIOTIC FLUID	1	120[2]	9	8	4	6
	2	200[2]	0	1	1	0
	3	200[2]	3	1	2	1

[1] HORMONE CONCENTRATION IN NG/ML

[2] APPROXIMATE DAY OF PREGNANCY BASED ON FETAL CROWN-RUMP LENGTH

* INSUFFICIENT QUANTITY OF SERUM FOR ASSAY

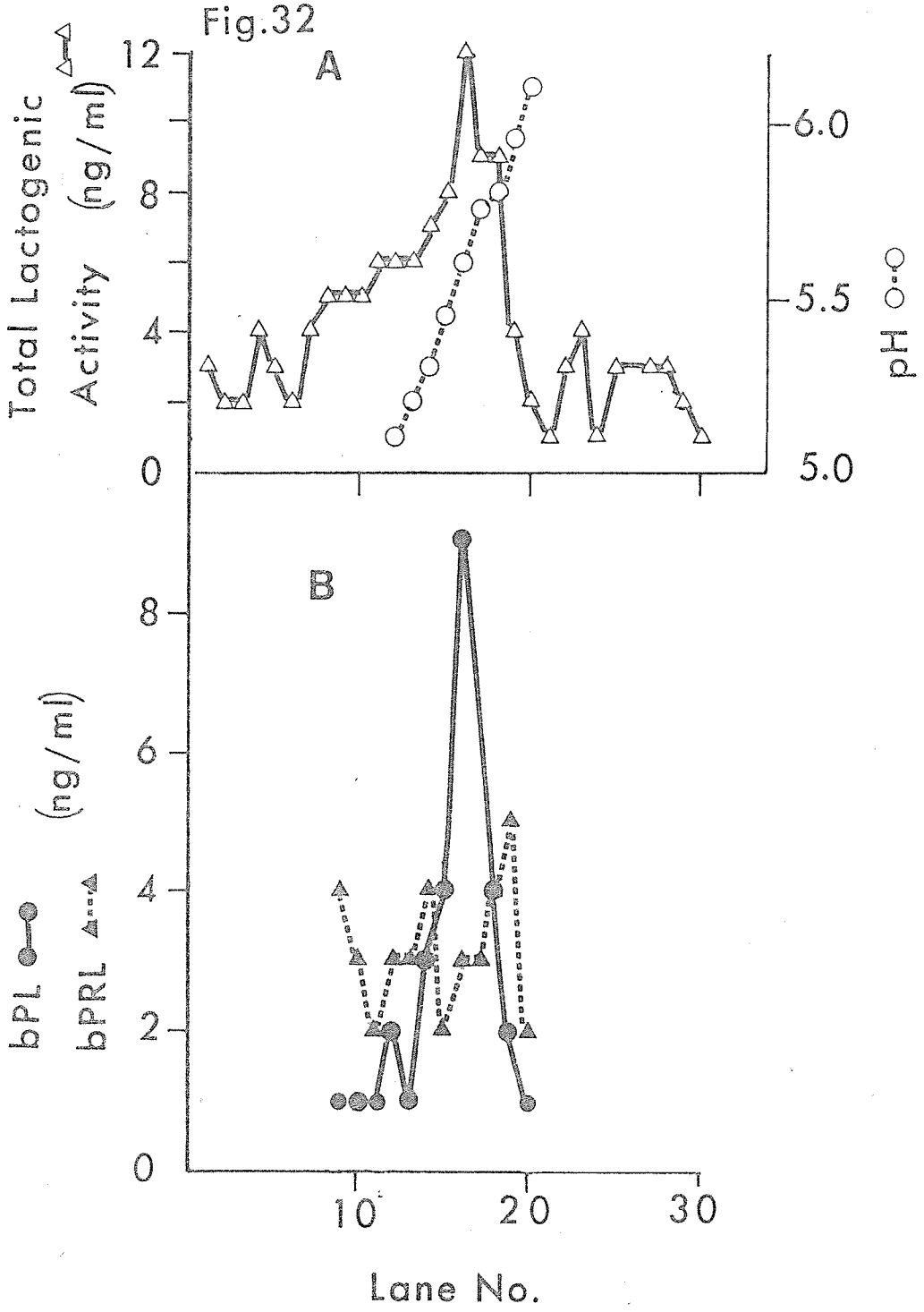
Figure 32

Distribution of lactogenic bioactivity in commercial fetal calf serum (FCS) following isoelectric focusing in a Sephadex gel system of pH range 4-6.5. Focusing was followed by the division of the gel into 30 lanes, determination of the pH profile by surface pH electrode, and elution of each gel lane with distilled water. Aliquots of the eluates were then assayed for lactogenic activity in the Nb2 lymphoma cell bioassay. Assay sensitivity is 1 ng/ml.

A. The left hand ordinate indicates the total lactogenic activity (△) of all eluates in ng/ml while the right hand ordinate demonstrates the pH profile (O) between lanes 11-20.

B. Lactogenic activity in eluate in the presence of excess antibody to bPRL (ie. residual activity attributed to bPL; ●) and in the presence of excess antibody to bPL (i.e. residual activity attributable to bPRL; ▲) is indicated in ng/ml on the ordinate.

Fig.32


A
B
Total Lactogenic Activity (ng/ml)
pH
bPL (ng/ml)
bPRL
12
8
4
0
6.0
5.5
5.0
6
2
10
20
30
Lane No.

V: DISCUSSION

A: PURIFICATION OF bPL

The choice of the RRA-hGH to monitor bPL during purification was based on the ability of the bovine hormone to displace ^{125}I-hGH from the rabbit liver membranes in a parallel (Figure 1) and equipotent (Figure 12) fashion to hGH. This fact combined with the availability of the assay reagents and the established use of the assay in numerous publications made the selection of this receptor assay straightforward.

The scraping of cotyledonary tissue from the fetal membranes was done to minimize the viscosity of the extract subjected to fractional ammonium sulfate precipitation. Selection of the 45%-55% fraction of the salt precipitation was made on the basis of the greatest amount of recovered receptor activity in the least viscous solution. The somewhat low recovery of activity during the gel filtration step is the result of the accumulated losses of a total of 5 column chromatographic procedures as explained in Materials and Methods.

The net result of the conventional purification scheme is that 2 % of the initial bPL is recovered after a 1000 fold purification.

B: PHYSICO-CHEMICAL CHARACTERIZATION OF bPL

Analytical acid disc polyacrylamide gel electrophoresis reveals the presence of a single stained band. The elution of bPL from a separate gel at a position

corresponding to the stained protein suggests that the purified hormone is in a homogenous state.

Analytical gel isoelectric focusing reveals the presence of only one apparent stained band (at pH 5.6; not shown) in the bPL preparation after focusing across a wide pH range indicating that the hormone preparation is homogeneous. Purified bPRL exhibits only one stained band as well. bGH displays several prominent protein bands. The single stained band seen for bPL and bPRL may be seen as representing homogeneity of these hormone preparations. However, this conclusion is qualified by the fact that the protein staining sensitivity of Coomassie Brilliant Blue is only about 1 ug; individual contaminating proteins of less than 1 ug/ml would not be visualized. Conversely the appearance of two or more closely associated bands may not necessarily indicate an impure hormone preparation, for individual proteins are subject to minor perturbations, such as deamidation, during purification and electrophoresis.

The Sephadex gel isoelectric focusing of a minimally purified placental preparation containing bPL, in the absence of an artificial pH gradient, suggests that, since most bPL was eluted at pH 5.5, the final purified product still exhibits essentially the same pI as it did upon initial extraction from placental tissue.

A comparison of the mobility of pure bPL in an SDS acrylamide gel with aliquots of material at various stages of purity allows the visualization of the effect

of purity allows the visualization of the effect of the various purification procedures on the removal of proteins other than bPL. Estimation of the molecular weight of pure bPL from this procedure is 35,000 daltons. Thus pure bPL obtained from fetal cotyledonary tissue has a molecular weight of about 35,000 daltons and electrofocuses predominantly at a pI of 5.5.

C: IMMUNOCHEMICAL EVALUATION OF bPL

The testing of the immunologic cross-reactivity of bPL with antibodies to various mammalian lactogenic and somatotropic hormones demonstrated that bPL is immunologically distinct from the human pituitary hormones PRL and GH, the bovine pituitary PRL and ovine GH, and also the rat or porcine PRL or rat or human PL. A significant degree of immunological relatedness is seen with ovine PL and a minor similarity to ovine PRL is detected. Evidence of this nature may suggest that structural (molecular) evolution of bPL occupies a position more intermediate between growth hormone and prolactin than does the apparent evolution of the primate placental lactogens (Bolander and Fellows, 1976).

D: BIOSYNTHESIS OF bPL BY BOVINE PLACENTA in vitro

A report by Forsyth and Buttle (1972) that bovine cotyledons from term placentas have lactogenic activity when co-cultured with mammary gland explants from mid-pregnant mice suggested that syntheses and secretion of bPL was possible by the bovine placenta.

The immunoprecipitation of a newly synthesized placental protein with the same apparent molecular weight as bPL confirms the ability of cotyledonary tissue to synthsize bPL *in vitro*. While evidence of secretion of bPL is not clear from this data, a recent report by Swanson and Beck (1980) confirms that *in vitro* placental cultures synthesize and secrete receptor active bPL.

E: RADIOIMMUNOASSAY FOR bPL

While the displacement curve for the placental extract parallels the standard curve for bPL, the non-parallel curves with pregnant cow serum and non-pregnant bovine serum and tissue extract samples indicate the presence of an interfering cross-reacting species detected in the RIA which is not bPL. Confirmation of the interference by this component in the RIA is evidenced in the fact that the RIA detects measurable levels of ^{125}I-bPL displacing material in steer serum and non-pregnant serum and milk. The values of apparent immunoactive bPL are elevated in the pregnant state but remain significantly lower than the levels reported by Bolander and Fellows (1976). The situation of tracer displacement by a molecular species other than bPL in the non-pregnant state and the apparent inability of the RIA to detect any truly elevated levels of 'bPL' in the pregnant cow has led to the assumption that the infidelity of one or another of the assay constituents was responsible for these perplexing results.

F: EVALUATION OF THE COMPONENTS OF THE RIA

Examination of the purity of the bPL employed as standards and tracer has been rather extensively conducted using the analytical biochemical techniaues described. The conclusion is that bPL is at least 90% pure with this estimate based on visual evidence demonstrating the presence of either one or a few closely associated stained bands in several polyacrylamide electrophoretic systems combined with the presence of receptor binding ability, coincident with the single protein.

Evaluation of the antigenic specificity of the antiserum generated to bPL showed that even pure bPL. with only a single component demonstrating an ability to bind to rabbit liver receptor sites following electrofocusing, contained two immunologically distinct and recognizable components. Visualization of an immunologically distinct but precipitable tissue factor which was not bPRL or bGH established that the antiserum to bPL contained an antibody population(s) generated towards an immunogen, present in many bovine tissues and all bovine sera, with the capability of displacing ^{125}I-bPL in the RIA. In fact, this component appears to form a line of complete identity in Ouchterlony gels with the precepitated antigen from placental extracts. The nature of this tissue component, beyond its immunologic similarity to bPL, is presently unknown. The possibility of establishing specificity in the RIA for bPL by the removal of the antibodies from the anti-bPL

antiserum which detect this tissue factor has been considered. The likelihood of this approach meeting with a successful conclusion may be diminished, however, because of the apparent identity between bPL and the tissue antigen.

The demonstration that anti-bPL, while precipitating predominantly the 35,000 dalton component of ^{125}I-bPL, also precipitates a lorger molecular weight tracer component may mean that the development of a functional RIA for bPL need only await the absolute purification of the bPL tracer (ie. the removal of this larger tracer component). Attempts to further purigy ^{125}I-bPL by gel exclusion chromatography, HTP chromatography, and ultrafiltration have to now, all failed to eliminate the displacement of tracer by non-pregnant serum.

Although excess anti-oPL antiserum was shown to precipitate ^{125}I-bPL, an attempt to develop a heterologous RIA for bPL by employing anti-oPL as the primary antiserum failed. The dilution of the antiserum to suit RIA conditions kiminished tracer binding to the exten that a standard curve for bPL could not be generated.

The existence of steroid binding proteins has provided the basis for several competetive binding assay systems for these hormones. A binding protein in the serum of the mouse has been shown to bind hGH tracer in the RRA for GH resulting in the estimation of falsely elevated levels of GH in mouse serum (Peters and Friesen, 1979). Such a binding protein for bPL in the cow could

be responsible for the appearance of apparent 'bPL' in the non-pregnant state. However, experiments designed to examine cow serum for the existence of such a bPL binding protein were unable to provide any indication of the existence of such a factor in the cow. The incubation of bPL in cow serum altered neither its elution profile upon gel filtration not its ability to bind to liver membrane receptors in the RRA for hGH.

Thus it appears that the 'bPL-like' immunological activity determined by the RIA-bPL in the non-pregnant and pregnant state is due to a high molecular weifht component of bovine serum (and presumably of numerous bovine tissues) which does not bind to somatotropic receptor sites.

Of much greater interest, however, was the discovery that the RRA-hGH was unable to detect any bPL in the serum of the late pregnant cow.

G: BIOLOGICAL CHARACTERIZATION

Equipotence of ^{125}I-bPL and ^{125}I-hGH in the RRA for hGH is evidence of the ability of the bovine placental hormone to bind to both the somatotropic and lactogenic receptor sites of rabbit liver membranes (Shiu et al, 1973). The binding and displacement characteristics of bPL in this receptor system are quite similar to those exhibited by oPL (Chan et al, 1976).

Binding of bPL to lactogenic receptors on rat liver membranes parallels that seen for pituitary prolactin from both the bovine and ovine species. The bovine

placental hormone, however, is only about 30% as efficient as oPRL in displacing ^{125}I-oPRL from the membrane receptors. Specific binding of the major radioactive component of bPL tracer by rat liver membranes and the demonstrated ability of excess hGH to selectively block binding of the tracer to the lactogenic receptors clearly indicates the lactogenic nature of the bovine hormone.

The similarity between the receptor binding characteristics of bPL and hGH, using rat and rabbit liver membrane preparations is clearly demonstrated. These hormones bind specifically to both lactogenic and somatotropic receptor sites with comparable ability. The overall binding and displacement characteristics of bPL are in contrast to those reported by Bolander and Fellows (1976) who found extremely low lactogenic and somatotropic receptor binding ability for their purified preparation of bPL. The results presented here are, however, in closer agreement to those reported by Hayden and Forsyth (1979) whose bPL preparation has a potency of 25 Iu/mg (equipotent to oPRL) in the lactogenic receptor assay.

The ability of bPL to significantly reduce the receptor concentration of human IM-9 lymphocytes confirms earlier reports of the ability of bovine placental extracts to display this characteristic (Lesniak et al, 1973) and now demonstrated that the active component in

the extract was bPL. These numerous observations indicate that bPL as oPL (Chan et al, 1976) has biological and perhaps structural and conformational features very similar to those of hGH.

H: BIOASSAY OF bPL

The lack of specificity of the RIA for bPL and the insensitivity of the RRA for hGH led to the search for a specific and sensitive assay for bPL capable of detecting the hormone in the circulation of the cow if it were present. The Nb2 node rat lymphoma cell culture bioassay, developed and characterized by Tanaka et al (1980), is specific for lactogenic hormones and has a sensitivity of as low as 0.4 ng/ml in serum.

The superimposable standard curves for bPRL and bPL indicates the equipotence of the two hormones with respect to their lactogenic properties in this assay. Antibody neutralization of homologous endogenous and/or exogenous hormone activity established the basis for use of this cell culture system as an appropriate bioassay for the lactogenic activity in bovine serum samples. The close agreement in measured levels of bPL hormone activity in the lactogenic and somatotropic receptor assays and the lactogenic bioassay provided evidence that the bioassay detected the same hormonal activity as the RRA used to monitor bPL purification. The similar assay values following electrofocusing also confirmed that bPL has a predominant pI of 5.5 with minor peaks of activity at pH 5.3 and 5.8.

Application of the Nb2 bioassay to the determination of the lactogenic bioactivity of bovine serum, milk, and amniotic fluid samples confirmed the suspicion raised by the data obtained with the RRA-hGH; no, or, at best, an extremely low level (≤ 3 ng/ml) of bPL exists in the peripheral circulation of the pregnant cow. All lactogenic activity is attributable to bPRL. This result stands in stark contrast to the bPL levels reported by Bolander and Fellows (1976) for pregnant cows in the second half of gestation. Buttle and Forsyth (1977) however were unable to detect any lactogenic activity in pregnant cow serum, other than that attributable to bPRL, using a rather insensitive mammary explant bioassay. The results of the more sensitive Nv2 assay confirm the observations reported by Buttle and Forsyth.

The Nb2 bioassay does, however, establish the existence of bPL in the circulation of the bovine fetus in the second trimester and the isoelectric point of the active bPL in commercial fetal calf serum corresponds to that obtained for pure bPL. Fetal bPL levels are in the same range as for oPL (Chan et al, 1978) in the sheep fetus. The process of obtaining uterine vein blood after slaughter of the cow involved a great deal of uterine manipulation and therefore raises the question of the origin and physiological significance of the bPL there.

The bPL levels detectable in amniotic fluid are presented with the same qualification as for the uterine

vein levels.

The situation of vastly different levels of placental lactogen circulating in the mother and fetus is known for several species including human, sheep, and monkey. However, in all these mammals, the levels of PL in maternal circulation, from the beginning of the second half of pregnancy, always exceed fetal levels. This report establishes the first demonstration of the lack of maternal PL in the face of a placenta synthesizing and apparently secreting the hormone into fetal circulation. Whether secretion of bPL into the cow's circulation takes place and what molecular changes are subsequently undergone by the hormone to cause loss of biological activity remains to be elucidated.

Clearly, active bPL is apparently destined only for the fetus. Suggestions that bPL acts as a maternal antiluteolysin (Rowson et al, 1979), or agent responsible for maternal response to altered metabolic demands in pregnancy, must certainly be reevaluated.

Examination of fetal and maternal tissue receptors, specific for bPL, may help to clarify the picture created by such unusual data.

The inability of a RIA for bPL to detect circulating hormone during pregnancy now seems not so strange. The development of monoclonal antibodies to specific antigenic determinants of the bPL may prove to be useful in studying the possivle degradation products of bPL in the maternal circulation.

CONCLUSION

This study outlines a purification procedure for bovine placental lactogen (bPL) from fetal cotyledonary tissue. A 1000-fold purification with a recovery of 2% was achieved.

Gel filtration chromatography, electrophoresis upon sodium dodecyl sulfate polyacrylamide gels, and isoelectric focusing suggest bPL has a molecular weight of 35,000 daltons with a pI of 5.5.

In radioreceptor assays for hGH (RRA-hGH) and oPRL (RRA-oPRL), bPL is equipotent to hGH and approximately one-third as potent as oPRL respectively. In the human IM-9 lymphocyte cell line hGH receptor modulation is observed with bPL. This result extends the evidence for structural similarity between hGH and the ruminant placental lactogens. In the Nb2 node rat lymphoma bioassay, bPL is equipotent to bPRL, further demonstrating the potent lactogenic characteristics of the hormone.

A homologous radioimmunoassay (RIA) for bPL was developed, but, even following extensive analysis and characterization of its components, the RIA detected the presence of a bPL-like factor in serum and tissue in the non-pregnant cows. These results negated a clear interpretation of immunoassayable bPL in pregnant cow serum samples. The inability to establish a functional (sensitive and specific) RIA for bPL has been one of the most perplexing aspects of this study. The use of

different preparations of pure bPL as standards and tracer, and several antisera, generated to bPL of varying degrees of purity (30-80%), has consistently led to the same unsatisfactory results. While the absence of bPL from the peripheral circulation of the pregnant cow undouvtedly confused the direction of endeavors to establish a competent RIA for bPL, the matter of the consistent appearance of an immuno-reactive component(s) in elevated concentrations in pregnant serum, similar to bPL and detected by several different antisera generated to bPL, presents the intriguing possibility that a cleavage product of bPL circulates in the dam. The generation of monoclonal antibodies to bPL, with the intention of selecting populations of cloned lymphocytes synthesizing and secreting antibodies directed against this component(s), would prove to be a rewarding exercise if it were demonstrated that even though lacking the typical biological effects of its precursor form (pure bPL), the circulating factor still possessed hormonal activity recognized by the cow.

The presence of bPL-like reactive sites is clearly shown in the immunohistochemical studies conducted with cryostat sections of bovine placental tissue. Biosynthetic studies on the <u>in vitro</u> incorporation of a radioactively labelled amino acid into newly synthesized and secreted placental proteins, showed the ability of fetal tissues to synthesize a protein speci-

fically recognized by antiserum to bPL and exhibiting identical mobility to ^{125}I-bPL in an SDS polyacrylamide gel. While substantial radioactivity in the incubation medium was immunoprecipitated by the antiserum, no direct evidence of bPL secretion is presented. Recognition of the anti-bPL antiserum characteristics, as demonstrated by Ouchterlony gel diffusion and RIA function, must be kept in mind when evaluating the localization and biosynthesis studies.

Finally, and most significantly, by applying a highly specific and sensitive bioassay to the measurement of lactogenic activity in bovine body fluids, there is strong evidence produced to substantiate the statement that bPL does not circulate in the peripheral blood of the pregnant cow. This data confirms and extends the previous <u>in vitro</u> bioassay results for pregnant cow serum (Buttle and Forsyth, 1976). These results are clearly contrary to those published by Bolander and Fellows (1976) where serum levels of bPL in dairy cattle are reported to be in excess of 1000 ng/ml. The reason(s) for the difference in molecular weight, potency, and circulating levels of bPL reported in this thesis and that presented in the last cited report is unknown.

VII: BIBLIOGRAPHY

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