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DEVELOPMENTALLY REGULATED CHANGES IN PROLACTIN mRNA AND GROWTH HORMONE mRNA LEVELS AND THEIR RESPECTIVE CELL TYPES IN THE FETAL BOVINE PITUITARY GLAND

presented by

Patricia Anne Fink

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Fritz M. Rottman
Major professor

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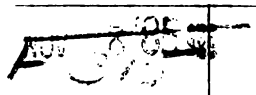

DEVELOPMENTALLY REGULATED CHANGES IN PROLACTIN mRNA AND GROWTH HORMONE mRNA LEVELS AND THEIR RESPECTIVE CELL TYPES IN THE FETAL BOVINE PITUITARY GLAND

By

Patricia Anne Fink

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ABSTRACT

DEVELOPMENTALLY REGULATED CHANGES IN PROLACTIN mRNA AND GROWTH HORMONE mRNA LEVELS AND THEIR RESPECTIVE CELL TYPES IN THE FETAL BOVINE PITUITARY GLAND

By

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Somatotrophs and lactotrophs, pituitary cells that produce growth hormone (GH) and prolactin (PRL), respectively, were localized in fetal bovine anterior pituitary glands using an immunocytochemical staining technique. Sagittal sections of the glands were scanned using a point-counting volumetric procedure to quantitate the volume percent of these two cell types as a function of development.

Total cellular RNA was extracted from fetal anterior pituitaries. Quantitation of PRL and GH mRNA levels were carried out by solution hybridization with single-stranded cDNA probes, generated from cloned, recombinant DNAs.

The concentration of GH mRNA and somatotrophs was high in fetuses of 3 months and older, and decreased twofold in the adult. PRL mRNA levels were low in the 3 month-old fetus, and increased sixtyfold during development; the number of lactotrophs increased fivefold. The expression of GH and PRL during development appears to be under different types of controls.

Dedicated to my parents and my sister.

ACKNOWLEDGEMENTS

I would like to thank Drs. H. J. Kung, E. M. Convey, and F. M. Rottman for serving on my guidance committee. I am especially grateful to Dr. Rottman for allowing me to pursue my interest in teaching while working on this project. Special thanks to Dr. John Nilson, in whose lab part of this work was done, for his encouragment and friendship.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
bGH	Bovine growth hormone
bPRL	Bovine prolactin
cpms	Counts per minute of radioactive material
EDTA	Ethylenediamine tetraacetic acid
GH	Growth hormone
GH-RH	Growth hormone-releasing hormone
GH-RIH	Growth hormone release-inhibiting hormone
hGH	Human growth hormone
IgG	Immunoglobulin G
PAP	Peroxidase anti-peroxidase complex
PRL	Prolactin
RDS	Respiratory distress syndrome
RIA	Radioimmunoassay
SDS	Sodium lauryl sulfate
SEM	Standard error of the mean
SSC	Buffer containing 0.15 M sodium chloride, 0.015 M sodium citrate
TRH	Thyrotropin-releasing hormone

LITERATURE REVIEW

The pituitary gland of mammals forms early in gestation and in most mammals, establishes its endocrine function before birth. Growth hormone (GH) and prolactin (PRL), two of the major pituitary peptide hormones, have been found in the pituitary gland of humans and ruminant animals by the second trimester [2, 3, 4]. The function of these hormones in the developing fetus has, however, remained elusive. The ontogeny of expression of growth hormone and prolactin, and the development of neuroendocrine regulation of these two hormones in the fetus, with emphasis on the bovine fetus, will be discussed here. A discussion of what is known of the function of these hormones in fetal development is also included.

The classical view of the origin of the anterior pituitary gland, or adenohyphysis, is that it develops as a diverticulum of the stomodeual epithelium, known as Rathke's pouch. This view had been challenged by recent evidence in avian embryos that the granular cells of Rathke's pouch may be derived from the ventral neural ridges, a portion of the primitive neural tube [5]. In bovine [6] and human fetuses [7], Rathke's pouch appears around the fourth to fifth week of gestation. (The duration of the gestational period of cattle and humans are very similar: human: 266 days; cattle: 280 days.)

Soon after the appearance of Rathke's pouch, parenchymal cells appear and begin to proliferate. Somatotrophs, glandular cells that produce GH, have been identified in pituitary glands from human fetuses as early as 9-10 weeks using differential histological staining [7], and 10 1/2 weeks using immunocytochemical staining [4]. Studies on the fetal bovine adenohypophysis did not reveal somatotrophs in fetuses of 3 months or younger by immunofluorescent antibody staining [2]. We have demonstrated the presence of somatotrophs in bovine fetuses as young as ten weeks [8], using the unlabeled peroxidase anti-peroxidase staining technique of Sternberger [1]. In the ovine fetus (gestation: 150 days), immunofluorescent localization of growth hormone in the anterior pituitary gland was first observed between 43 and 53 days [3]. In these three species the somatotroph first makes its appearance by the end of the first trimester of pregnancy.

Lactotrophs, cells that produce PRL can first be found in the fetal bovine anterior pituitary gland of 14 weeks [8], in the human fetus of 14 weeks [66] and in the ovine fetus of 11-12 weeks [3]. Thus the fetal pituitary glands of these mammalian species have the capacity to synthesize and store these protein hormones by the second trimester.

In adult animals, secretion of PRL and GH are under hypothalamic controls. Prolactin secretion has been shown to be primarily under inhibitory control by dopamine, a catecholamine made in the hypothalamus and released into the

hypothalamic-hypophyseal portal system [10]. Thrytotropin-releasing hormone (TRH) has also been shown to influence PRL secretion; it appears to stimulate release of PRL from secretory granules. Its role in fetal PRL secretion appears to be minor, and will not be discussed here. For a general review see Gluckman et al. [11]. The secretion of growth hormone appears to be under both negative and positive control by the hypothalamic regulatory hormone, growth hormone-releasing hormone (GH-RH) and somatostatin, also called growth hormone release-inhibiting hormone (GH-RIH)[12]. Fetal plasma and pituitary content of GH and PRL are probably a reflection of the degree of maturity of these neuroendocrine control mechanisms.

Pituitary PRL content [13] and plasma concentration [16] increase between 90 days and the end of term in the bovine fetus. In ovine [14, 11] and human fetus [15] plasma and pituitary content show the same general trend as in the bovine fetus.

The concentration of GH in the pituitary gland of bovine fetuses are high at 90 days, sixfold higher than the concentration of PRL in a similarly aged fetus. There is a fourfold increase in pituitary GH concentration by term [13]. Plasma concentration of this hormone are also high during gestation and decrease at birth [16]. The pattern of fetal pituitary concentration of GH is similar in sheep and humans [11]. Circulating plasma GH levels in the ovine fetus exhibit a bimodal curve, while in the human, fetal

levels peak at midgestation and fall at term [18]. Nonetheless, in all three species, pituitary and plasma GH levels are relatively high at some point during fetal development and decrease later in the neonate and/or adult.

Hypersecretion of GH in the fetus has been postulated by Kaplan et al. to be due to the immaturity of central nervous system control of pituitary function [18]. "The ontogeny of GH secretion progresses from a hypersecretory phase induced by unrestrained GH-RH stimulation during fetal development to a stage of partially controlled release in infancy and finally to the regulated release of GH-RH secretion as affected by the circulating levels of GH, steroids, adrenergic receptors and higher central nervous system responsiveness" [18].

Experiments with stalk-sectioned ovine fetuses have indicated that during the second trimester, central nervous system stimulation of GH secretion is present [20]. This procedure, in which the pituitary stalk connecting the pituitary to the hypothalamus is severed, caused a rapid fall in plasma GH levels. Thus a hypothalamic factor, presumably GH-RH, appears to be influencing GH secretion in the fetus.

Somatostatin, or GH-RIH, is a cyclic tetradecapeptide that has been found in the hypothalamus, stomach, gut, pancreas, and brain [12]. Somatostatin inhibits the secretion of GH and has been found by radioimmunoassay to be present in hypothalamic tissue of ovine fetuses as early as 60 days [21]. Higher levels are found later in gestation. Infusion

of somatostatin in catheterized fetal lambs suppresses the secretion of GH, thus demonstrating the presence of receptors on the fetal somatotroph [22].

Both positive and negative hypothalamic controls of GH secretion appear to be present in ruminant fetuses. Gluckman et al. [11] postulated that the stimulatory effect of GH-RH on GH secretion is predominant in midgestation, while the inhibition by somatostatin becomes increasingly important later in the development of the animal.

Clinical observation of human fetuses suggest, however, that these controls are not fully mature [11]. In the adult, secretion of GH is influenced by many stimuli, including psychic factors, endogenous sleep rhythms, exercise and a host of stressful, hormonal and metabolic controls [24]. Signals of these events are transmitted to the hypothalamus; presumably they affect the secretion of somatostatin or GH-RH in some manner [25]. Many of these stimuli have no effect on GH secretion in human neonates.

The control of plasma PRL levels, in contrast, is mainly under negative control in adults. To investigate development of dopaminergic regulation of PRL secretion in ovine fetuses, Gluckman et al. [26] infused the dopamine antagonist haloperidol into chronically catheterized ovine fetuses. In the late gestation fetus, haloperidol caused an immediate increase in plasma PRL levels, suggesting that inhibition of PRL secretion by dopamine is active in the fetus. In the same study, use of dopamine agonists, such as

apomorphine and bromoergocryptine, indicate that the ability of fetal lactotrophs to respond to dopamine is present by 106 days, prior to the rapid increase in the plasma PRL levels seen between 110 and 120 days. This implies that the rise in plasma levels of this hormone is not due to the lack of maturation of dopaminergic control.

The effect of estrogen on PRL secretion has been demonstrated in a number of species, including the rat, ovine, bovine and human [28, 29, 30, 31]. In ovine fetuses, the increase in plasma PRL levels is preceded by an increase in estrogen levels [27]. To test the hypothesis that estrogen is responsible for the increase in PRL levels seen in late gestation, Gluckman et al. [11] infused 17-βestradiol into chronically catheterized ovine fetuses at 90 or 105 days of gestation. A twofold increase in PRL secretion was observed in fetuses of 105 days but not at 90 days.

In cows, the estrogen effect on PRL secretion has been shown <u>in vivo</u> and <u>in vitro</u>. Bovine pituitary cells secrete more PRL into the culture mediawhen incubated with estradiol [31]. <u>In vivo</u> experiments by Schams and Karg have also demonstrated a dose dependent increase in plasma PRL concentration, in response to estradiol [32]. Thus estrogen may be responsible for the pattern of PRL levels seen during gestation in the ruminant fetus.

Estrogen is known to inhibit the effect of dopamine on lactotrophs in rats [33]. The rise in PRL levels during gestation may be due to the effect of high circulating

estrogen levels of placental origin, which would negate the inhibitory effect of dopamine on PRL secretion [71].

Additional evidence for the lack of hypothalamic regulation of PRL secretion in fetal animals comes from studies on human anencephalic infants, and ovine stalk-sectioned fetuses. In anencephalic infants, the neural tube fails to develop normally, and although a pituitary gland may be present, normal hypothalamic-hypophyseal interactions are not. PRL concentration in plasma and the pituitary gland are normal in these infants, as are the numbers of lactotrophs [11]. Also, stalk sections in ovine fetuses late in gestation fail to affect plasma PRL levels [34]. Experimental evidence thus far indicates that the mechanism for dopamine inhibition is present in the developing fetus, and that rising estrogen levels may be responsible for the pattern of PRL secretion observed in the fetus.

The function of PRL and GH in the developing fetus has not been well defined. PRL has been implicated in the development of the fetal lung and it may also have an osmoregulatory effects on fetal and amniotic membranes. GH, interestingly enough, appears not to be required for fetal somatic growth. It may stimulate adrenal steroidogenesis, and there is evidence that, in the rabbit fetus, GH affects the functional metabolic development of the fetal liver.

PRL may play a role in development of fetal lungs by affecting surfactant synthesis. During the normal development of the lung, special cells in the alveolus begin to

produce surfactant, a complex phospholipid whose major active component is dipalmitoyl-phosphatidylcholine. Surfactant is an important component of the fluid lining the alveoli, and is responsible for the low surface tension at the air-tissue interface, preventing collapse of the alveoli during expiration. The importance of surfactant in normal pulmonary function is best illustrated in the respiratory distress syndrome (RDS). RDS is a leading cause of mortality in premature infants; death is due to inadequate levels of surfactant which causes the collapse of the air sacs and pulmonary insufficency [35].

PRL has been postulated to stimulate surfactant synthesis, in concert with other hormones [36]. High affinity receptors for PRL have been found cell membranes of lung tissues in fetal rhesus monkeys. Results indicated that a fetal lung cell population may have more affinity for PRL than an adult cell population from the lung [37]. Studies on the concentration of PRL in fetal cord blood in relationship to the incidence of RDS in newborns have demonstrated that premature infants who developed RDS had significantly lower cord serum PRL levels than their counterparts [38]. These lines of evidence are all suggestive of a role of PRL in the appearance of surfactant in fetal lung development.

More direct evidence comes from <u>in vitro</u> studies by Hamosh and Hamosh [36]. Rabbit fetuses injected with 1 mg of ovine PRL at 24 days of gestation showed increased levels of lung phospholipid, lecithin and dipalmitoyl lecithin

within two days. These findings have, however, been questioned by other investigators, who were unable to demonstrate any effects of PRL on phospholipid levels [39] or maturation of pulmonary function in fetal rabbits [40]. Gluckman et al. [11] and Giannopoulos and Tulchinsky [41], in reviews on the subject, have concluded that PRL at most has an indirect effect on fetal lung development.

A second possible role of PRL in the fetus may be regulation of water and electrolyte balance in fetal and amniotic membranes. PRL has been shown to have osmoregulatory actions in most vertebrate animals [42]. Friesen et al. have speculated that PRL may be required for sodium transport mechanisms in fetal membranes analogous to the function of PRL in lower animals [43]. They have reported that the concentration of PRL in amniotic fluid is 100 to 200 fold greater than either fetal or maternal serum levels during gestation. The souce of these high levels appears to be maternal [44], although there are conflicting reports [11]. Josimovich, et al., using rhesus monkeys, have demonstrated that administration of PRL into the amniotic fluid during the last trimester of gestation caused a decrease of 50% in amniotic fluid volumes [45]. Replacement of the amniotic fluid with hypertonic or hypotonic fluid caused an increase or decrease, respectively, of water and electrolyte content of the fetal extracellular fluid volume. These fluxes were prevented if PRL was injected into the amnion. They speculate that the protective effect of PRL in the face

of altered tonicity might be important when the amniotic fluid becomes hypotonic to serum when fetal urine excretion becomes significant late in pregnancy.

The lack of effect of GH on fetal somatic growth was most strikingly demonstrated in the rabbit fetus by Jost [46]. Rabbits decapitated in utero during gestation were normal in body length at birth, compared to their litter mates. In humans, there is no clinical evidence that GH plays a role in somatic growth [11]. Size is normal in infants congenitally lacking a pituitary gland; there is lack of correlation of plasma GH levels with birth weight; and premature infants do not respond to hGH. In the rat there are certain strains that are genetically dwarfs. These rats are deficient in GH and PRL [48], but are normal in size at birth [47].

In cattle there is a report that Guernsey calves with adenohypophyseal aplasia are smaller than normal in size and weight at birth [49]. Electrocoagulation of the pituitary gland in fetal sheep during the latter part of gestation caused a retardation in growth as judged by body weight [50]. In these species, GH may already be necessary for normal growth before birth [51]. In a recent review, however, Gluckman et al. [11] concluded that growth retardation in these studies was due to fetal hypothyroidism, and that, in general, there was little evidence to support a role of GH in fetal somatic growth.

Another possible role of GH in fetal life may be the stimulation of adrenal steroidogenesis. Devaskar et al. have reported that, in late gestational catheterized lambs, infusion of pharmacological levels of GH caused plasma corticosteroid concentrations to increase between 60 and 350% above basal levels. In vitro studies with fetal rabbit adrenal cortical cells also revealed a similar effect of GH secretion on corticosteroids [52]. In addition to its role in adrenal steroidogenesis, Jost has also proposed that GH stimulates glycogen deposition in the liverof fetuses, in concert with adrenal corticoids [53].

In conclusion, the anterior pituitary gland becomes functional early in development in cattle and other mammals. Peptide hormones such as PRL and GH are found in the circulation as early as the end of the first trimester, and come under neuroendocrine regulation later in development. The role of these hormones in development is uncertain, and may be species specific.

INTRODUCTION

The fetal bovine pituitary gland contains a high concentration of GH throughout development. The amount of PRL in the gland is low early in gestation and increases during the latter part of development [13]. The amount of PRL mRNA also increases during development [9]. The present study was undertaken to examine further the expression of GH and PRL in the fetal bovine pituitary gland. The levels of the mRNAs for these two hormones, and also the volume percent of the differentiated cell types that produce each hormone were quantitated throughout gestation, in order to evaluate the level at which control of expression exists.

MATERIALS AND METHODS

Collection and preparation of tissues

Bovine fetuses were obtained at Murco Co., Plainwell, Michigan, over a three-year period. Anterior pituitary gland were dissected free of the posterior pituitary gland and membranes, and placed immediately in Bouin's fixative [54]. The smallest fetuses (2-3 cm crown to rump length) were fixed intact. Crown to rump length and sex of each fetal animal were recorded. Gestational age of each fetus was estimated by its crown to rump length, according to methods described by Benesch and Wright [55]. Neonatal pituitary gland were obtained from male calves of less than one month of age, bought from farmers in central Michigan. Anterior pituitary glands of these calves were cut mid-sagittally and one-half placed in liquid nitrogen, and the other in Bouin's fixative. Pituitary glands were obtained from adult animals Houghton's slaughterhouse, Ionia, Michigan. Tissue to be used for RNA hybridization studies was frozen immediately in dry ice.

Anterior pituitary glands and whole embryos were fixed in Bouin's fixative for 24-48 hours, depending on the size of the tissue. The tissue was washed in 50% (w/v) ethanol, dehydrated in graded concentrations of ethanol, cleared in xylene or toluene, and embedded in paraffin [54]. Serial sections of four micrometers in width were made in

the sagittal plane, these sections were individually collected and sequentially numbered. Five to ten sections from each of three areas in the anterior pituitary gland were affixed to glass slides: sections from the midsagittal area, and sections in an area one quarter of the sagittal thickness of the entire tissue on both sides of the midsagittal plane.

Immunocytochemistry

A modification of the unlabeled peroxidase-antiperoxidase method was used to localize lactotrophs and somatotrophs in tissue sections [1]. Pituitary sections were deparaffinized, washed, and then preincubated in a saline solution containing 3% normal goat serum (Polysciences, Inc.), 0.2% Triton X-100, 0.05 M Tris-Cl, pH 7.5, for thirty minutes at 4^{o} C. Sections were then exposed in succession to rabbit anti-bovine PRL serum or rabbit anti-bovine GH serum (dilutions 1:1000, 1:500 respectively), goat anti-rabbit IgG (dilution 1:10, Polysciences, Inc.), and peroxidase anti-peroxidase (PAP) complex (dilution 1:50, Polysciences, Inc.). Rabbit antisera to bovine GH was kindly provided by Dr. A. F. Parlow, Director, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, California. Sections were incubated with the primary antisera, rabbit sera to bovine growth hormone (bGH) or prolactin (bPRL), for a minimum of two hours, and with the remaining antisera for thirty minutes, all at 4^{o} C. All antisera were diluted in a saline solution containing 1%

normal goat serum (Polysciences, Inc.), 0.2% Triton X-100, and 0.05 M Tris-Cl, pH 7.5. Sections were washed three times between successive treatments with antisera, using Tris-buffered saline (0.05 M Tris-Cl, pH 7.5, 9% NaCl). The final incubation step to produce the red-brown reaction product involved treating the sections with 0.25 mg/ml 3,3' diaminobenzidine (Sigma Chemical Co.), 0.003% H_2O_2 in 0.05 M Tris-Cl pH 7.5 for four minutes at room temperature. Sections were counterstained using Harris' Alum Hematoxylin, dehydrated, and mounted in Permount.

To visualize both somatotrophs and lactotrophs in the same tissue section, the procedure described by Erlandsen et al. was used [59]. Sections were stained for the first antigen using diaminobenzidine as the chromagen, as previously described. The sections were then incubated in 1.0 N HCl, to remove the unreacted antibody molecules. Rinses in distilled water were followed by a ten minute incubation with Tris-buffered saline containing 10% normal goat serum. Incubations with antisera to the second antigen, goat anti-rabbit IgG, and the PAP complex were as described. 4-Chlorol-napthol (ICN Pharmaceuticals) was used as the second chromagen, at a concentration of 0.8 mg/ml. The 4-chloro-1-napthol was dissolved in 0.5 ml of dimethylformamide, then added to 10 ml of 0.05 M Tris-Cl, pH 7.5 and 1 ul of 30% H_2O_2; the sections were stained for four minutes, washed and mounted in glycerol.

Preparation and characterization of primary antisera

Rabbit anti-bovine prolactin serum used in the immunocytochemical staining was obtained from an immunized New Zealand white male rabbit, three to five years of age. To immunize the rabbit, two to three mg of bovine prolactin, dissolved in alkaline distilled water pH 10 and mixed with Freund's adjuvant, were injected subcutaneously at two week intervals. Three days after the third injection, the rabbit was bled by the ear, the blood allowed to clot, and the serum collected and frozen. Three weeks later a booster injection of one to two mg of bPRL was given. Again, the animal was bled from a marginal ear vein, the serum collected and frozen. To characterize the antisera, Ouchterlony immunodiffusion plates were run. Diluted immune serum was tested against saline, bovine prolactin, and bovine growth hormone. No precipitin line was observed against a 0.75 mg/ml solution of bGH, while a specific line was formed against solutions of bovine PRL ranging in concentration from 0.01 to 0.5 mg/ml. Nonimmune pooled rabbit sera was also tested; no precipitin lines were formed against bGH or bPRL. Bovine growth hormone and prolactin were provided by the National Institute of Arthritis, Metabolism and Digestive Diseases.

Radioimmunoassays (RIA) were run in the laboratory of Dr. Edward Convey, Department of Animal Science, Michigan State University. Serum collected after a booster injection of bovine prolactin bound 33% of the total cpms of

^{125}I-PRL at a dilution of 1:10,000; at a dilution of 1:15,000 the antisera bound specifically 19% of the iodinated bovine prolactin. Serum collected from the first bleeding did not show any precipitable counts above background at a dilution of 1:10,000 or greater, and therefore was not used in any of the immunocytochemical staining.

Immunocytochemical control procedures

The following control procedures were conducted to assess the specificity of the staining procedures:

a) nonimmune pooled rabbit sera was substituted for the primary antisera

b) 0.05 M Tris-Cl buffered saline was substituted for the primary antisera

c) staining intensity was evaluated by running simultaneous staining reactions with increasing dilutions of the primary antisera up to a dilution at which staining was no longer detectable.

No reaction product was observed with these control procedures.

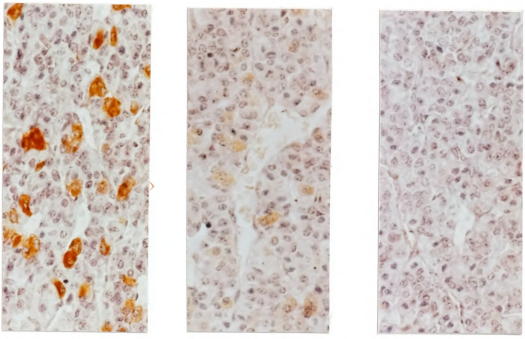
Antibody specificity of both primary antisera was tested under the actual conditions used in the immunocytochemical staining. The primary antisera was diluted 1:1000 in a saline solution containing 1% normal goat sera, 0.2% Triton X-100, and 0.05 M Tris-Cl, pH 7.5. One milliliter of this diluted antisera was then incubated with varying amounts, 0.1 to 30 ug of bPRL or bGH, at 4^{o} for 24 hours.

The solution was centrifuged for ten minutes in a microfuge to pellet any antibody-antigen complexes. The resulting supernatants were then used in place of the primary antiserum in the immunocytochemical staining protocol already described. The results of these experiments are shown in Figure 1. Adsorption of rabbit anti-bovine prolactin with bPRL abolished all staining at a concentration of 20 ug/ml. Adsorption of the GH antisera with 1.0 ug bGH removed over 90% of all specific staining.

To test whether there was any cross staining under conditions used in the staining procedure, adsorption experiments similar to those described above were done. Rabbit anti-bovine GH was adsorbed with varying amounts, 0.1 to 50 ug, of bPRL; the prolactin antisera was adsorbed with similar ranges of bGH, and both were processed as described above. There appeared to be no effect on the specificity or intensity of the immunocytochemical staining (Figure 2).

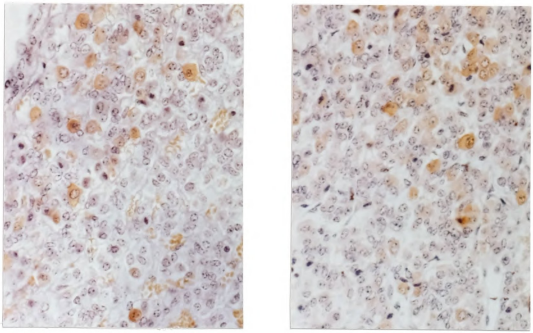
Quantitation of somatotrophs and lactotrophs

The volume percent of lactotrophs and somatotrophs were determined on three sagittal sections per individual from each of the three areas described, essentially according to the point counting volumetric procedure described by Chalkley [56]. A nine-point stereological test probe was placed in the 10X eyepiece of a compound microscope, and fields were counted using an oil immersion objective at a magnification of 1000. Each tissue section was scanned and a systematic pattern of fields were counted. A point was



(A) (B) (C)

Figure 1. Immunospecificity of anti-bPRL staining in a fetal bovine anterior pituitary gland. (A) Section incubated with anti-bPRL 1:1000 showing characteristic staining of cells. Remaining serial sections were incubated wtih anti-bPRL 1:1000 premixed with bPRL at the following hormone concentrations: (B) 10 ug/ml; (C) 20 ug/ml. Diminution of cell staining is detectable when anti-bPRL is absorbed with bPRL at 10 ug/ml and staining disappears as the hormone concentration is elevated. X100.



(A) (B)

Figure 2. Lack of cross-reactivity between anti-bGH and bPRL (A) Section of fetal bovine pituitary gland exposed to anti-bGH 1:1000 showing characteristic staining of cells. (B) Serial section was incubated with anti-bGH 1:1000 premixed with bPRL at a concentration of 50 ug/ml. X100.

counted as a hit when it fell over any portion of an immunostained cell. All points falling over stromal elements were counted, and the volume percent of immunochemically stained cells was expressed as a percentage of total glandular parenchymal volume. An average of 1000 $\pm$ 100 points per tissue sections was counted (range 185-2856).

To determine if the total number of field counted per section constituted a sufficient sample, preliminary counting of a section was done as follows. The section was systematically scanned and counted several times; each time an increasing number of fields were analyzed. The data were then plotted to show the volume percent as increasing numbers of fields in a systematic pattern were analyzed. The cumulative volume percent thus plotted showed less and less variation as the number of measurements increased. From this graph, an empirical estimate of the size of the sample necessary for a reliable measurement was determined for animals of similar ages.

Isolation of RNA

Total cellular RNA was prepared from anterior pituitary glands of adult, neonatal, and fetal cattle as described by Nilson et al. [9]. Briefly, this procedure involved homogenization of the tissue in a proteinase K, SDS-containing buffer, using a loose Teflon motor-driven pestle in a glass barrel. This suspension was incubated for two hours at 45^{o} C, made 0.1 M in salt,and extracted with phenol and chloroform. One gram of CsCl was added per ml of

aqueous phase, and was then layered onto pads of 5.7 M CsCl in SW 50.1 nitrocellulose tubes. Centrifugation at 35,000 rpm for at least 16 hours at 22° C allowed precipitation of the RNA, while leaving the DNA in the supernatant. The RNA pellets were washed and ethanol precipitated.

Preparation of single-strand cDNA probes

cDNA clones for bGH and bPRL were isolated from a bovine pituitary cDNA library and characterized as described [8, 9]. Plasmid DNA was labeled by the procedure described by O'Farrell et al. [57]. To separate the cDNA strands, poly A^+RNA was hybridized in excess to the cDNA at 50° C overnight. Treatment with S1 nuclease degraded all unprotected RNA and DNA sequences. The hybridized mRNA was removed from its complementary DNA by hydrolysis with NaOH. For a more complete description of these procedures see Nilson et al. [8].

Blotting and hybridization of RNA

Two micrograms of total cellular RNA were electrophoresed in 1.5% agarose gels containing 0.02 M borate pH 8.3, 0.2 mM EDTA, and 3% formaldehyde as described [58]. Electrophoresed RNA was transferred to Gene Screen (NEN) using the conditions specified by the supplier. Hybridizations were carried out for 48-72 hours at 45° C in 50% formamide, 0.02 M Pipes, pH 6.4, 0.8 M NaCl 0.002 M EDTA, 0.5% SDS, 100 ug/ml sheared and denatured salmon sperm DNA, and 50,000 cpm/ml of single-stranded DNA. The filters were then washed twice in 2X SSC-0.17% SDS at room temperature

and twice in 0.1% SSC-0.17% SDS at room temperature. The hybridized RNA was detected by autoradiography with Kodak XAR-5 film, using a Dupont Cromex Intensifying screen at-80° C.

Solution hybridization of RNA to cDNA in excess

Hybridizations were carried out in either 50% or 80% formamide containing 0.4 M NaCl, 0.01 M Pipes, pH 6.4, 0.001 M EDTA, 50 ug/ml poly A, 0.19% SDS and 5000 cpm of single stranded cDNA. A volume of 20 ul was used for the hybridization reaction with 50% formamide, while a volume of 80 ul was used with 80% formamide. In either case, the temperature of hybridization was 45° C. All hybridization reactions were carried out under mineral oil in sterile, disposable polypropylene tubes (Falcon 2063). The extent of hybridization was measured by S1 nuclease treatment as described previously [9].

RESULTS

Lactotrophs

Lactotrophs are distributed throughout the bovine anterior pituitary gland of adults, but are especially abundant in the peripheral regions. Higher volume percents were found in sagittal sections from the lateral areas of the gland as compared to that of the midsagittal section (Table 1). In sagittal sections, there was a marked lack of lactotrophs in a region medial and anterior in the gland; this area was referred to as the basophilic zone (Figure 3). Lactotrophs appear in clusters through out the gland and these cells are polygonal in shape (Figure 4). The distribution of lactotrophs in the neonatal male calves was very similar to that found in adult animals; the volume percent of these cells was lower in the neonate compared to fetal or adult levels.

Lactotrophs were not observed in 12 fetuses younger than three months of age, or 15 cm crown to rump length. Glands studied from fetuses between 3 and 4 1/2 months showed increasing numbers of lactotrophs; seven glands taken from fetuses between 4 1/2 and 7 1/2 months showed similar volume percents to each other, in both lateral sagittal and midsagittal sections. The two anterior pituitary glands looked at from animals older than 7 1/2 months of

Table 1. Volume Percent Lactotrophs as a Function of Fetal Age

Fetal Age	Volume percent lactotrophs[a]		Number of animals	Sex
	Lateral sections	Medial sections		
1-3 months	0	0	5 embryos 7 fetuses	undetermined 3M,4F
3-4½ months	6.6 ± 2.2	3.3 ± 0.4	4	2M,2F
4½-7½ months	15.3 ± 0.4	6.7 ± 0.4	7	3M,4F
>7½ months	20.0 ± 5.0	9.2 ± 2.8	2	1M,1F
Neonates	7.1 ± 1.8	3.3 ± 0.7	2	2M
Adults	39.5 ± 0.6	25.0 ± 0.5	4	2M,2F

[a]Results are expressed as mean ± SEM

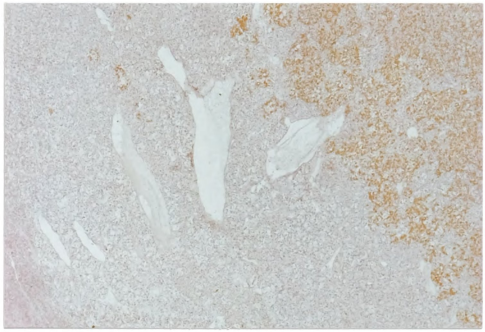


Figure 3. Zonation of lactotrophs in the adult bovine pituitary gland. Lactotrophs are stained reddish-brown; section has been counterstained with hematoxylin. Note the lack of lactotrophs in region on left, and high concentration to the right. These areas are referred to as the basophilic and acidophilic zones, respectively. X10.

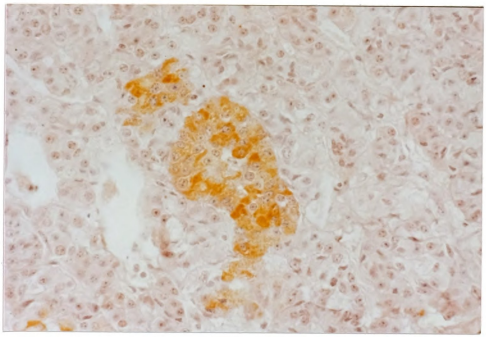


Figure 4. Clustering of lactotrophs in the adult bovine pituitary gland. Section has been stained using the immunocytochemical technique of Sternberger [1], and counterstained with hematoxylin. X100.

gestational age exhibited a greater volume percent of lactotrophs than the previous age groups (Table 1).

Fetal lactotrophs first appeared singly, and by 5 months appeared in clusters similar to that found in adult animals (Figure 5). There appeared to be no change during development in the size of the lactotrophs relative to other cells in the gland.

Somatotrophs

Bovine somatotrophs, cells that produce GH, exhibit a pattern of distribution similar to that of lactotrophs, in that they are totally absent from the basophilic zone. In contrast to the lactotrophs, however, somatotrophs appeared to have a uniform distribution throughout the rest of the pars distalis. Somatotrophs tended to be round cells, and were distributed as single cells throughout the acidophilic area of the anterior pituitary gland (Figure 7).

Five embryos between the ages of 1-2 months were immunochemically stained for GH; no immunostained cells were found in the sagittal sections examined. Anterior pituitary glands from fetuses of 10 cm crown to rump length or greater, the next age group examined, contained a great abundance of somatotrophs (Table 2). The numbers of somatotrophs in the three sagittal sections examined per animal remained relatively high throughout fetal life. Somatotrophs in the same tissue section showed a variation in the intensity of immunostaining; some cells stained darkly, while others were stained lightly.

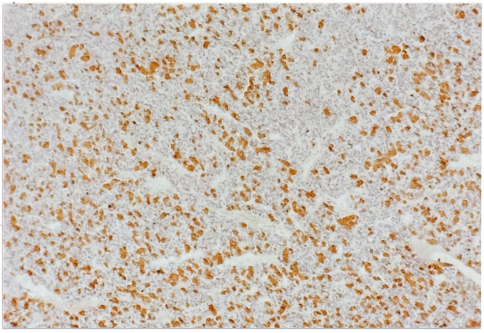


Figure 5. Lactotrophs in the anterior pituitary of a bovine fetus of 5 months. Immunostained cells (reddish-brown) show a tendency to be arranged in groups. X25.

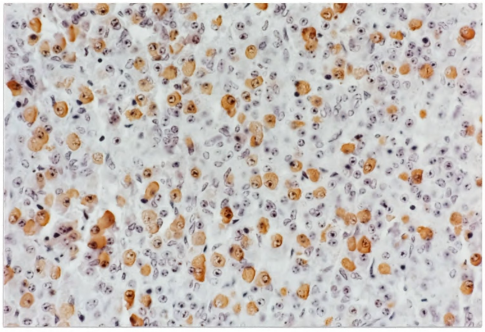


Figure 7. Somatotrophs in the anterior pituitary of an adult cow. Immunostained cells (reddish-brown) are distributed single throughout section. Note the generally round or oval shape of these cells. X100.

Table 2. Volume Percent Somatotrophs as a Function of Fetal Age

Fetal Age	Volume percent somatotrophs[a]		Number of animals	Sex
	Lateral sections	Medial sections		
<2 months	0	0	5 embryos	
2-3 months	20.3 ± 4.3	9.0 ± 2.1	3	1M,2F
3-4½ months	50.6 ± 4.3	23.8 ± 3.3	3	1M,2F
4½-7½ months	47.7 ± 3.2	23.7 ± 1.5	4	2M,2F
>7½ months	42.0 ± 3.0	23.0 ± 2.1	2	1M,1F
Neonates	21.2 ± 1.25	10.4 ± 1.0	2	2M
Adults	20.6 ± 3.2	11.0 ± 2.4	4	2M,2F

[a]Results are expressed as mean ± SEM

The two neonatal pituitary glands contained numbers of GH cells similar to that found in adult anterior pituitary glands.

In order to facilitate a comparison between changes in mRNA levels and cell numbers as a function of development, the changes in volume percents was plotted using a bar graph (Figure 9). Because the size of the lactotrophs or somatotrophs appeared not to change relative to the other differentiated glandular cells in the pituitary throughout development, changes in volume percents will be equated with changes in cell numbers.

During development of the bovine pituitary gland, some cells of the pars distalis become associated with the intermediate lobe of the pituitary. These cells are separated from the remainder of the anterior pituitary gland by the hypophyseal cleft; this area is known as the cone of Wulzen, described by Wulzen in 1914. In the study presented here, this area of the gland was not included in any of the quantitations, due to the fact that in the separation of the posterior lobe from the anterior, these cells are removed with the posterior pituitary gland. In a male fetus of 2 1/2 months of age, the entire gland was removed and stained for somatotrophs. As can be seen in Figure 6, there are somatotrophs present in the cone of Wulzen. An adjacent section was stained for lactotrophs; there were no cells present that contained PRL.

Figure 9. Percentage of lactotrophs and somatotrophs as a function of gestational age. Data redrawn from Tables 1 and 2. Data of volume percents in the lateral section only were used to simplify the presentation; the relative pattern of change of lactotrophs and somatotrophs as a function of development in the medial section is similar to that observed in the lateral sections. Solid bars represent the mean value of volume percent lactotrophs; cross-hatched bars represent mean value of volume percent somatotrophs. Plunger bars indicate the SEM.

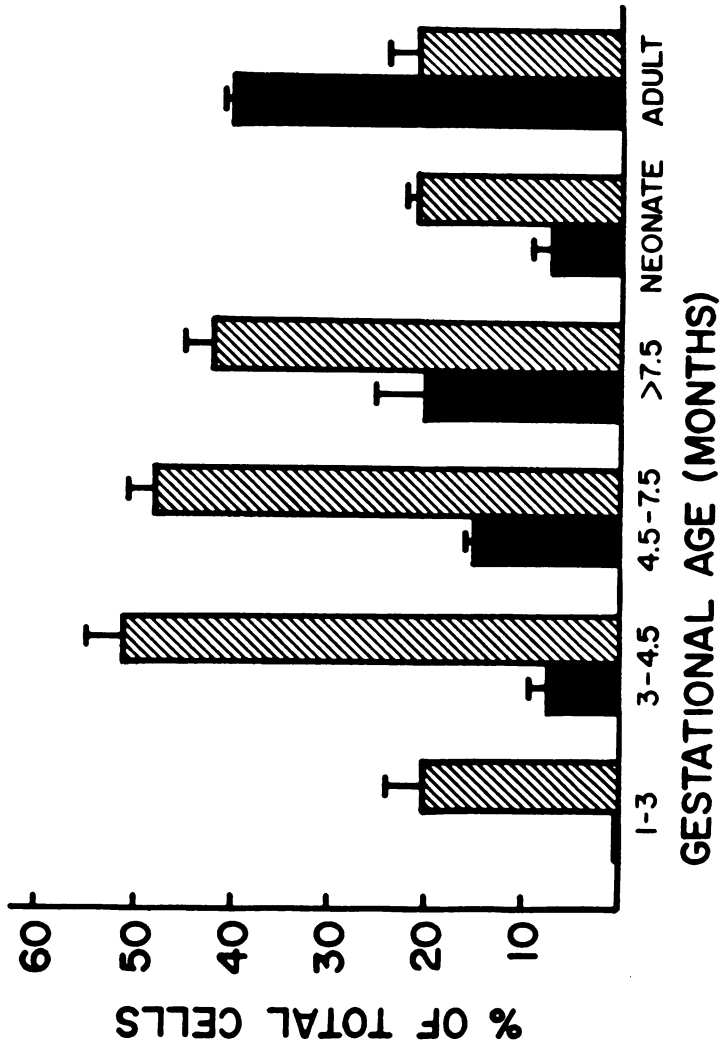

% OF TOTAL CELLS
60
50
40
30
20
10
1-3
3-4.5
4.5-7.5
>7.5
NEONATE
ADULT
GESTATIONAL AGE (MONTHS)

Figure 9

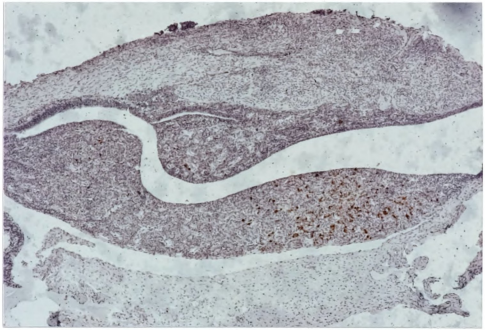


Figure 6. Somatotrophs in the pituitary of a bovine fetus of 10 1/2 cm, showing the cone of Wulzen. Cells that have stained more intensely with hematoxylin represent glandular cells of the anterior pituitary and cone of Wulzen. The knob-like group of cells projecting in to the hypophyseal cleft is the cone of Wulzen. Somatotrophs, cells stained reddish-brown, are present in both areas, though in greater abundance in the anterior pituitary. X10.

Lactotrophs and somatotrophs as two distinct cell populations

Bovine fetuses of 2, 16, and 42 cm crown to rump length were stained for both lactotrophs and somatotrophs in the same tissue section, as were sections from a neonatal pituitary gland and a female adult pituitary gland. In none of the sections thus stained were any cells present that stained for both proteins within the same cell (Figure 8). Adjacent sections stained for PRL and GH also demonstrated the lactotrophs and somatotrophs as two distinct cell populations.

Relative GH and PRL mRNA levels

Levels of GH and PRL mRNAs in the fetal, neonatal and adult pituitary gland were determined using solution hybridization to single-stranded cDNA, as described [8], and were expressed as a percentage of the adult mRNA levels. The concentration of GH mRNA is at least 30 times greater than PRL mRNA in fetal or neonatal pituitary glands, whereas in adult pituitary glands, PRL mRNA levels are approximately two times greater than GH mRNA levels. The estimate of a two-to-one ratio of PRL mRNA to GH mRNA in adult glands was made by a variety of methods. For a more complete description see Nilson et al. [8]. The concentration of GH mRNA increase only gradually during gestation, then decline after parturition; in the adult the concentration of GH mRNA is at least twofold lower than at any time during fetal life. In contrast, fetal PRL mRNA levels are extremely low early in

Figure 8. A neonatal bovine pituitary gland stained by the unlabeled immunoperoxidase double staining method [59]. Cells containing growth hormone (gray) are different from cells containing prolactin (black). X200.

Figure 8

development, then increase over sixfold in concentration by parturition, and rise by another factor of ten at some time after the neonatal period (Figure 11). The results from Northern transfers demonstrate that there is no gross alteration in the size of the GH mRNA or PRL mRNA throughout development (Figures 11, 12).

Figure 10. Relative levels of GH mRNA and PRL mRNA as a function of gestational age. Total cellular RNA isolated from individual fetal or neonatal anterior pituitaries was analyzed for content of PRL mRNA and GH mRNA using solution hybridization to single-stranded cloned cDNA as described in text. Each fetal age group represents a minimum of four animals; only three neonatal pituitaries were analyzed. Adult levels of each mRNA were determined from pooled pituitaries from a large number of animals. Fetal and neonatal mRNA levels were expressed as percent of adult levels. A minimum of a two-to-one ratio of PRL mRNA to GH mRNA in the adult was estimated from results of in vitro translation assays and other evidence. Solid bars represent the means of levels of PRL mRNA; cross-hatched bars, means of GH mRNA levels.

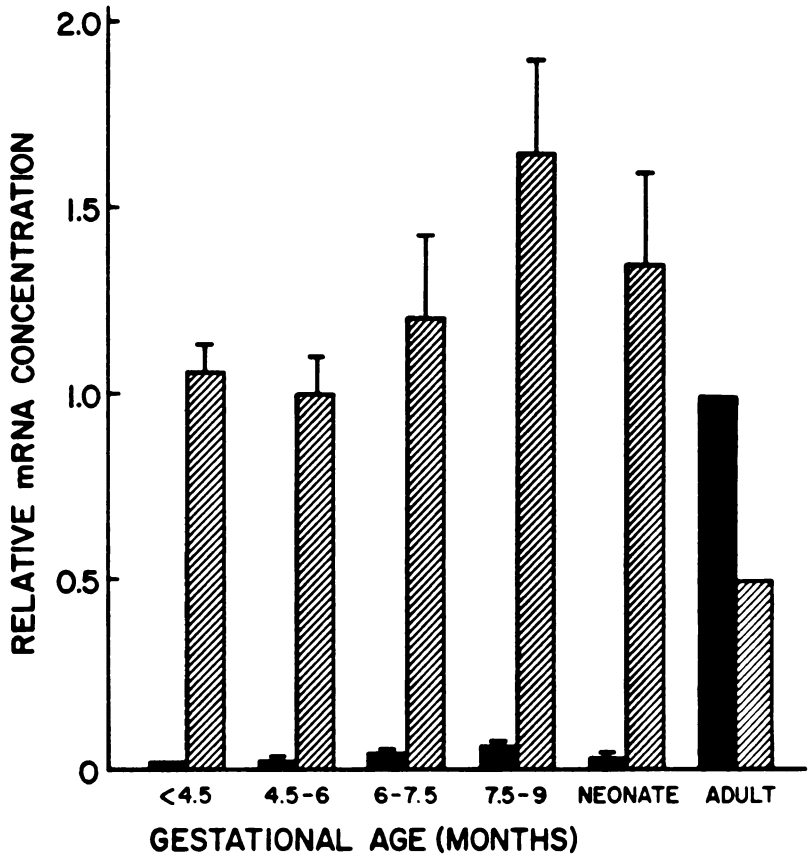

2.0
1.5
1.0
0.5
0
RELATIVE mRNA CONCENTRATION
<4.5
4.5-6
6-7.5
7.5-9
NEONATE
ADULT
GESTATIONAL AGE (MONTHS)

Figure 10

Figure 11. Northern transfer of fetal RNA: hybridization with GH cDNA. Equal amounts of total cellular RNA were electrophoresed in 1.5% agarose-formaldehyde gels, transferred to Gene Screen, and hybridized to GH cDNA, as described in text.

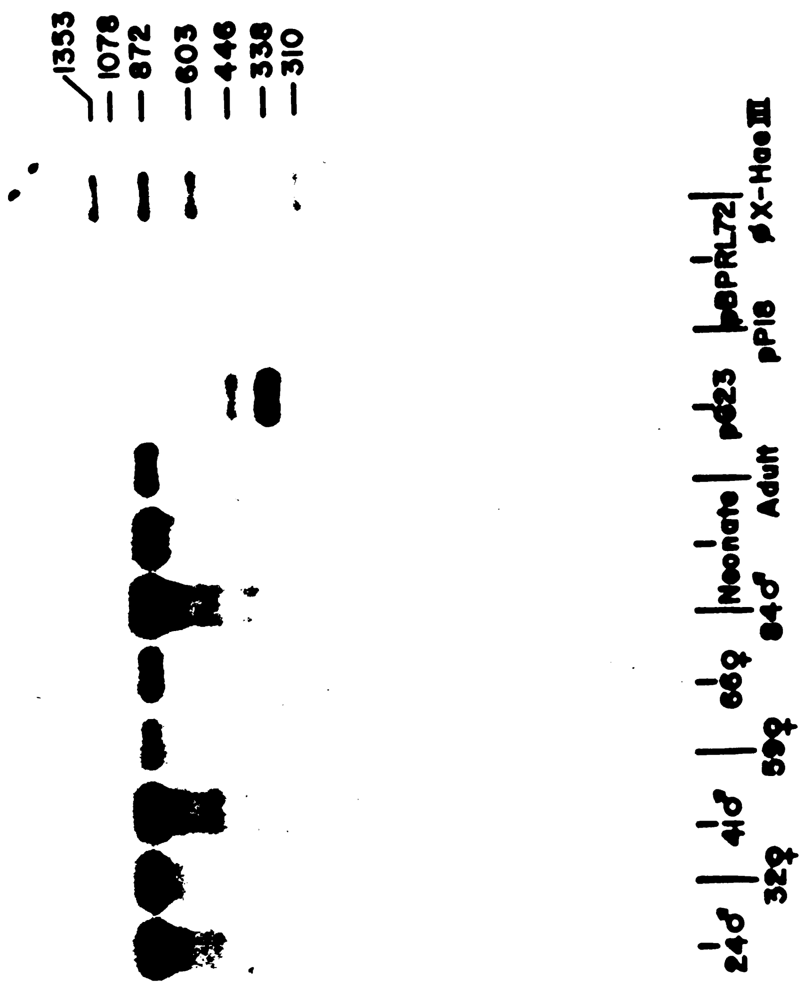

1353
1078
872
603
446
338
310
24♂
32♀
41♂
59♀
68♀
84♂
Neonate
Adult
p623
pPI8
pBPRL72
φX-HaeIII

Figure 11

Figure 12. Northern transfer of fetal RNA: hybridization with RPL cDNA. Equal amounts of total cellular RNA were electrophoresed in 1.5% agarose-formaldehyde gels, transferred to Gene Screen, and hybridized to PRL cDNA, as described in text.


1353
1078
872
603
446
338
310

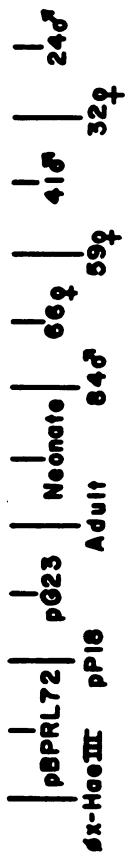

pBPRL72
6x-HaeIII
pG23
pP18
Neonate
Adult
84♂
66♀
59♀
41♂
32♀
24♂

Figure 12

DISCUSSION

Immunocytochemical techniques have been used to reveal the presence of GH and PRL in separate cells of the anterior pituitary glands of a number of animals, including cattle, sheep, and deer [66, 3, 60]. The fetal pituitary glands of these animals have also been studied. Stokes and Boda have localized GH and PRL in anterior pituitary cells of fetal lambs [3]. Menghellia and Scapinelli have described somatotrophs in fetal calves [2]. We have extended these studies in the bovine fetus. We have described fetal lactotrophs in addition to somatotrophs in bovine fetuses and neonates, and have attempted to quantitate these cell types as a function of development.

Studies of the morphometric analysis of the pituitary gland of ungulates using immunocytochemical staining methods are limited. Schulte et al. [60] have described seasonal changes in the volume density of lactotrophs in the white-tailed deer. They observed increased volume density of lactotrophs during midsummer, but concluded that these changes were most readily explained by volume changes in, rather than hyperplasia of, pre-existing cells. Quantitative studies of the cell types in the anterior pituitary glands of cattle have been reported by Bassett [61]. Using

histological staining techniques that differentiate the glandular cells of the adenohypophysis into three types, acidophils, basophils and chromophobes, she reported that the numbers of acidophils (GH- and PRL-producing cells) were lower in cows with cystic ovaries compared to the normal cows. Lactotrophs and somatotrophs have been reported to represent approximately 40-50% of the cell population of the anterior pituitary gland as reported for the human [62] and sheep [63].

Reports on the changing numbers of these cells during development have been at best, semi-quantitative. In pituitary glands from human fetuses, Pavlova et al. [64] reported the first appearance of acidophils at 11-12 weeks, with a rapid increase between 12 and 20 weeks, and cells numbers levelling off by parturition. Lactotrophs appeared at 14 weeks of gestational age and remained few through 16 1/2 weeks. Their numbers increased greatly at 23 weeks [65]. Both of these reports fit well with data reported here. There appears to be a primary event early in development that stimulates rapid proliferation of the committed cell type. Throughout the rest of prenatal life, the rate of proliferation of the lactotrophs and somatotrophs levels off to a lower level.

Available cytological and immunocytochemical evidence supports the view that PRL and GH are synthesized and stored in separate cells in the pituitary gland. The question of separate identities of lactotrophs and somatotrophs in the

pituitary gland of cattle and related species has been investigated by a number of workers [66, 3, 67]. Generally, the approach has been to look for identical cells in thin adjacent sections that have been stained for both GH and PRL. The use of double staining immunocytochemistry has made the approach and analysis simpler by avoiding the use of adjacent sections.

During the cytodifferentiation of the pituitary gland, it is believed that a precursor cell type, capable of producing both GH and PRL, gives rise to the two differentiated cell types, the somatotroph and lactotroph. Evidence for the existence of this stem cell was first demonstrated in GH_3 cells lines derived from rat pituitary tumors [68].

Human acidophil stem cell adenomas have been described which also contain cells believed to be a neoplastic form of the stem cell of lactotrophs and somatotrophs [69]. These tumors contain cells that produce PRL and GH, usually in the same cell, as shown by immunoperoxidase staining technique.

Shiino et al. [10] have described clonal cells derived from a multipotential clone of fetal rat cells from Rathke's pouch; these subclones secrete both PRL and GH. They have proposed a scheme for the cytodifferentiation of anterior pituitary cells, in which a multipotential stem cell gives rise to a progenitor cell of the lactotroph and somatotroph cell lines, and a progenitor cell of the glycoprotein hormone-secreting cells and the committed ACTH-producing cell. A similar scheme of cytodifferentiation has been

proposed by Schecter from electron microscope observations of the rabbit anterior pituitary gland [71].

In the present study, an attempt was made to identify cells in the pituitary gland of bovine fetuses that produce both PRL and GH. Sections of glands from fetuses of different gestational ages were stained for PRL and GH, using the double staining technique described by Erlandsen et al. [59]. In none of the sections thus stained was there evidence of cells that contained both antigens. The youngest fetuses were approximately 1 1/2 months of gestational age. Staining for PRL or GH was not observed in these fetal glands. Because only a few sections of these glands were examined, it cannot be stated conclusively that the anterior pituitary gland at this stage of development did not contain stem cells of the type described by Shiino [70]. A better tissue fixative and modifications of the immunocytochemical staining technique would have to be made in order to detect the low levels of antigens that would be present in progenitor type stem cells. An alternate approach would be to use in situ hybridization techniques to localize the specific mRNA in ultrathin tissue sections [79].

The primary purpose of this study was to look at the expression of PRL and GH during prenatal development in cattle. A tenfold increase in the percentage of PRL mRNA to total cellular RNA had been found to occur between 90 and 200 days of gestation, with a further 20 fold increase in the adult gland [9]. Thus the question was raised whether

the increase in the levels of PRL mRNA during development represented an increase in the numbers of cells that produce PRL or increased transcriptional activity of the gene. We were also interested in the expression of GH in fetal bovine pituitary glands; the results of these studies are reported here and elsewhere [8].

The results indicate that during development and maturation of cattle, level of PRL mRNA increases as a result of proliferation of lactotrophs and increased expression of the PRL gene. As shown in Figure 9, the percent of lactotrophs increases approximately twofold from the latest fetal group to the adult animal. The concentration of PRL mRNA, however, increases about fifteenfold over the same time span. This implies that, in addition to proliferation of the lactotroph, there must be an increase in the transcriptional activity of the PRL gene at this time. In contrast, there does not appear to be any developmental modulation of GH gene activity in the bovine.

In the neonatal pituitary glands studied, the levels of the PRL mRNA and lactotrophs fell compared to fetal levels. A similar pattern has been found in the early neonatal lamb [14, 16]. This may be a reflection of lower estrogen levels due to the removal from influence of the placental steroids.

In contrast, levels of GH mRNA remained high, while somatotrophs appeared to decrease in their relative proportions to other glandular cells of the pituitary. The decrease in volume percent of somatotrophs may be more

apparent than real. mRNA levels were measured on only three pituitary glands from neonates; cell counts were done on only two of those three glands. Another possible explanation could be that, as the neonatal period is a period of rapid growth, the secretory granules may be releasing their contents at a much higher rate than in the fetal period. Stored GH as detected by the immunocytochemical staining might only represent a portion of the somatotroph population.

There are numerous reports on the effects of hormones on the proliferation and gene activity of lactotrophs. The major regulators of PRL, estrogen and dopamine, appear to have an effect at both levels.

Reports of the effects of estrogen on the expression of PRL are numerous. There are fewer reports, however, that include an effect on changes in cell numbers due to estrogens. Gersten et al., using estrogen implants placed into the pars distalis of ovariectomized rats, detected a direct effect of estrogen on the cells of the anterior lobe [72]. Using immunocytochemical staining to identify the lactotrophs, they detected proliferation of these cells in the area of the implant; the cells also appeared to increase in size.

In a developmental study using immature rats, Maurer noted that treatment with estradiol resulted in elevated serum prolactin levels, and increased cellular division in the pituitary gland. He concluded that the developmental

changes in secretion of PRL are probably induced by altered estrogen concentrations, and that the estrogen probably also caused a proliferation of the lactotroph [73].

The effects of estrogen on expression of PRL in sheep have been shown by Shupnik et al. [74] and Vician et al. [75], *in vivo* and *in vitro* using primary cell cultures. In ovariectomized ewes treated with a synthetic estrogen, translatable levels of PRL mRNA were increased above control levels. In vitro experiments using primary ovine pituitary cell cultures have also demonstrated the effect of estrogen on levels of translatable PRL mRNA. They conclude that in the sheep estrogen can directly cause an increase in both the number and gene expression of lactotrophs. Recent evidence, using immunocytochemistry to detect lactotrophs in cell cultures, have confirmed their conclusions [80].

In humans, the proliferation of lactotrophs in the maternal adenohypophysis is also accompanied by increased levels of PRL in the serum. This is also thought to be a direct result of high estrogen levels [70].

The effect of dopamine on PRL secretion has already been discussed. Maurer, using rat pituitary cells has been able to demonstrate that dopamine inhibits the synthesis of PRL and production of PRL mRNA [77]. When autologous pituitary grafts are placed beneath the kidney capsule of hypophysectomized rats, to remove the pituitary from hypothalamic influences, PRL secretion increases, as does the number of lactotrophs [78]. Thus dopamine, like estrogen,

appears to exert its effect at the level of cellular proliferation and gene expression.

Because dopamine and estrogen have been found to be important regulators of PRL levels of adult animals of other species, and both appear to affect cellular proliferation and gene activity of the lactotroph, they are primary candidates for hormones that affect PRL expression during the development and maturation of the bovine pituitary gland. Whatever the mechanism may be, there is precedent in the literature for factors that affect both cellular proliferation and increased transcription of a specific gene in a differentiated cell.

The control of GH secretion appears to be due to the effects of somatostatin and GH-RH. The effects of these factors on the cellular proliferation of somatotrophs has not been reported in the literature.

The evidence presented here and elsewhere [8], indicates that during the development of the bovine pituitary gland, increased PRL levels are due to both proliferation of lactotrophs and increased expression at the gene level. Thus is appears that in the fetal bovine pituitary gland, the PRL gene is not maximally expressed at the time it is first turned on. The GH gene in contrast, appears to be maximally expressed once it is turned on. The significance of differential controls of gene expression for PRL and GH during development are not known.

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