The University of Manitoba

THE ONTOGENESIS OF GLYCOPROTEIN HORMONES AND THEIR α AND β SUBUNITS IN BOVINE FETAL PITUITARY GLANDS

by
Jaroslaw V. Workewych

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree Master of Science.

Department of Physiology
Winnipeg, Manitoba
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I wish to dedicate this thesis to my mother, father, sister and grandmother.

ABSTRACT

A membrane bound receptor pellet was obtained from fresh porcine thyroid glands after homogenization and differential centrifugation. Highly purified bTSH (30 i.u./mg) was used as standard, and labelled with ^{125}I for tracer. The binding of ^{125}I-bTSH was dependent on the concentration of membrane protein in the incubation mixture. Optimal specific binding was obtained at pH 7.0 after 1 hour incubation at 37° or 4 hours at 25°. A radioreceptor assay for TSH has been developed with a sensitivity of 10-20 ng/ml. The binding of ^{125}I-bTSH was specific and not displacable by other protein or polypeptide hormones up to concentration of 10,000 ng/ml. The TSH activity detected in the pituitary extracts from cattle, dog, rabbit, rat, guinea pig, turkey and chicken pituitary glands interacted in a parallel manner to bTSH standard. The molecular size of TSH showed an inter-species similarity, but electrophoretic mobilities varied slightly between species.

Sensitive homologous radioimmunoassays have been developed for bFSH, bFSHα and bFSHβ. Antisera were raised with New Zealand white rabbits. The sensitivity of the RIA for bFSH was 1 ng/ml, and the cross-reactivities of all protein and polypeptide hormones tested were $<$ 1%. In the RIA for bFSHα, preparations of bFSHα, bLHα and bTSHα displaced equally on a weight basis, starting at a

concentration of 1ng/ml, showing that the three α-subunits were immunologically identical. Bovine FSH, bLH and bTSH all cross-reacted identically at a level of 10%. The cross-reactivities of all other protein and polypeptide hormones tested were < 1 %. The RIA for bFSH was sensitive to a level of 0.5 ng/ml. Intact bFSH cross-reacted at a level of 5%. The cross-reactivities of all other hormones tested were <1%.

Intact glycoprotein hormones and free α-subunit activity detected in bovine fetal pituitary extracts were separable by gel filtration on Sephadex G-100. The elution volumes of these activities in pituitary extracts were identical to the purified standards. The relationship between age of fetuses (months) and wet weight of fetal pituitary glands (mg) was found to be linear. Pituitary glands from males $<$ 3 3/4 months gestational age showed elevated free α-subunit levels when compared to pituitary glands from female fetuses of the corresponding age group. There is a significant increase of bFSH in pituitary glands from female fetuses $>$ 5 months gestational age. Free bFSHβ, bLHβ and bTSHβ were not detectable in bovine fetal pituitary extracts. The concentrations of bFSH, bLH, bTSH and free α-subunit per pituitary gland increased with fetal age; however the ratio of free α-subunit: total glycoprotein hormones per pituitary gland decreased. Free α-subunit from bovine fetal pituitary glands was similar in molecular size to bFSHβ, bLHβ and bTSH; however, these α-

subunit preparations exhibited slight variations in electrophoretic mobilities. The free α-subunit from bovine fetal pituitary glands cross-reacted in a parallel manner to bFSHα, bLHα and bTSHα in the RIA for bFSHα showing that all four were immunologically identical; however the free α-subunit did not recombine with bFSHβ indicating some functional differences between the free α-subunit in pituitary glands and the α-subunits obtained by dissociating intact hormones.

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LIST OF ABBREVIATIONS

Hormones

LH	luteinizing hormone
FSH	follicle-stimulating hormone
TSH	thyroid-stimulating hormone
LHα	the α subunit of luteinizing hormone
LHβ	the β subunit of luteinizing hormone
FSHα	the α subunit of follicle-stimulating hormone
FSHβ	the β subunit of follicle-stimulating hormone
TSHα	the α subunit of thyroid-stimulating hormone
TSHβ	the β subunit of thyroid-stimulating hormone
GH	growth hormone
PRL	prolactin
Ins	insulin
HCG	human chorionic gonadotropin
HCGα	the α subunit of human chorionic gonadotropin
HCGβ	the β subunit of human chorionic gonadotropin
ACTH	adrenocorticotrophic hormone
TRH	thyrotropin releasing hormone
LHRH	luteinizing hormone releasing hormone

Prefix Denoting Species of Origin

b	bovine
o	ovine
p	porcine
r	rat
h	human

Assays and Activities

RRA	radioreceptor assay
RIA	radioimmunoassay
RAS	rabbit antiserum
specific binding	CPM bound (e.g. to receptor) that can be displaced by excess hormone
% specific binding	$\frac{\text{CPM specifically bound}}{\text{total CPM}} \times 100$

Reagents

^{125}I	prefix denoting radioactively iodide labelled molecule (e.g. ^{125}I-bLH)
PBS	phosphate buffer with saline (0.9% w/v)
BSA	bovine serum albumin

Gel Filtration and Polyacrylamide Gel Electrophoresis

V_o	void volume
R_f	electrophoretic mobility = $\frac{\text{distance of migration by protein}}{\text{total distance migration by ion-front}}$

Units of Measure

g	gram
mg	milligram
μg	microgram
ng	nanogram
ml	milliliter
μl	microliter
W/V	weight per unit volume (g/100 ml)
cm	centimeter
mm	millimeter
nm	nanometer
M	molar
mM	millimolar
N	normality
in	inch

Miscellaneous

S.D.	standard deviation
p	probability
g	unit of gravitational force

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INTRODUCTION

The Glycoprotein Hormones

The glycoprotein hormones are the class of hormones made up of protein with attached carbohydrate groups. In vertebrates, three glycoprotein hormones, follicle-stimulating hormone (man, cattle, rat, sheep and swine), luteinizing hormone (man , cattle, dog, monkey, rabbit, rat, sheep and swine) and thyroid-stimulating hormone (man, cattle, goat, rabbit, rat sheep and swine) are secreted by the adenohypophysis of the pituitary gland.

The principal biological effect of FSH is to stimulate the growth and maturation of ovarian follicles.

Luteinizing hormone was named for its ability to induce the formation of the corpus luteum. In females, LH is required for the corpus luteum to secrete its hormones, estradiol and progesterone. Luteinizing hormone also acts synergistically with FSH in promoting follicular development. In males, LH is required for spermiogenesis by the semeniferous tubules and for the biosynthesis of testosterone by the Leydig cells.

The principal role of TSH is to stimulate the synthesis and secretion of the thyroid hormones, thyroxine and triiodothyronine.

A fourth glycoprotein hormone, chorionic gonadotropin (CG) has to date only been detected in humans and monkeys. Chorionic gonadotropin is secreted by the trophoblastic cells coincidently with ovum implantation and by the

chorionic villi of placenta tissue during the first trimester of pregnancy. Chorionic gonadotropin is chemically and functionally similar to LH. The function of CG is that of a luteotropin, capable of maintaining the early corpus luteum of pregnancy and prolonging luteal secretory activity beyond the menstrual cycle. This extension of luteal function precludes menstrual sloughing and ensures proper implantation and nourishment of the conceptus until the placenta is able to secrete estradiol and progesterone in sufficient quantities to maintain pregnancy.

Radioimmunoassays

A radioimmunoassay is based on the ability of a hormone, by simple competition, to inhibit the binding of a radioa-ctively labelled hormone to its specific antibodies. Antibodies are produced by immunization of animals of another species, usually rabbits, guinea pigs and sheep, with the hormone against which the antibodies are desired. The hormone concentration of an unknown sample is determined by comparing the degree to which it inhibits the binding of radioactively labelled hormone by the antibody, to the degree of inhibition produced by a series of standards containing known amounts of the hormone.

Radioimmunoassay techniques have developed over the years as a result of the observations first made in 1956

by Berson and Yalow (54) on the interaction between insulin and insulin binding antibodies. Since that time, antibodies have been produced against a great number of polypeptide hormones (55). Radioimmunoassays for hFSH (57), hLH (43), hTSH (56), HCG (61,64), hFSHβ (60,61), hLHβ (59), hTSHβ (58), HCGβ (43) as well as the common α-subunit of the human glycoprotein hormones (58,59,60, 62,63) have all been developed and characterized.

Owing to the presence of the common α-subunit in LH, FSH, TSH and HCG there are problems of immunological cross-reactivities in the radioimmunoassays for glycoprotein hormones.

Radioreceptor Assays

The preparation of highly purified polypeptide hormones, the availability of a simple procedure for producing radio-iodinated hormones (45) and the development of method for the separation of cellular components (46) were the technical achievements that led to the direct study of polypeptide hormone-receptor interactions.

Radioreceptor assays are based on the principles of competitive binding. The radioactively labelled hormone binds to the receptor and can be displaced by the unlabelled hormone competing for the same binding sites. Radioreceptor assays are specific as biologically dissimilar hormones will not displace the radioactively labelled hormone from its target receptor.

In 1970, Lefkowitz et al. (47) developed the first radioreceptor assay of ACTH using membranes from the adrenal gland as the target receptor. Since then, radioreceptor assays for other polypeptide hormones including all of the glycoprotein hormones, have been developed. Radioreceptor assays for FSH have been developed using membrane fractions from rat testes as the target receptor by Means and Vaitukaitis in 1972 (48), Bhalla and Reichert in 1974 (49), and from bovine testes by Cheng in 1975 (50). Radioreceptor assays have also been reported for LH by Catt et al. (51), for HCG by Kammerman et al. (52) and for TSH by Winand and Kohn (53).

Radioreceptor assays detect biologically similar molecules; whereas radioimmunoassays detect immunologically related molecules.

The Biochemistry of Glycoprotein Hormones

The glycoprotein hormones LH, FSH, TSH and HCG, have been shown to consist of two dissimilar polypeptide chains (1,2,3). The common subunit, designated α, has an identical amino acid sequence within a species (4,5,6,7, 8,9,12) whereas the β subunits are distinct from each other and are responsible for the biological and immunological specificities of each hormone (6,7,8,10,11). The hormone-specific β subunits within a species can be aligned so that about 50% of their sequences are homologous (12).

It has been shown by many researchers that although the structural integrity of both subunits is less rigid for subunit-subunit recombination, structural integrity is generally necessary to generate the full biological activity of the intact hormone. It has been shown that nitration of the tyrosine residues of the β chains of bLH (74,75) and oLH (14) results in normal recombination with the α chains of bLH and oLH, respectively, whereas nitration of the tyrosine residues of the αchains of bLH (74,75) and oLH (14) results in only 20-25% recombined product. Modification of the carboxyl group of bLHα by incorporation of 8 residues of glycine methyl ester, results in the normal recombination with the bLHβ chain giving rise to normal bLH activity (15), however modification of the carboxyl groups of bLHβ by incorporation of 7 residues of glycine methyl ester, yields a partially active bLH upon recombination with the intact bLHα subunit (15). Acylation of the free amino groups in the oLHβ subunit results in normal recombination with the oLHα subunit; however, the activity of the oLH generated is 33-50% less than that of recombining native oLHα and oLHβ (16). Cheng et al. showed that after removal of carboxyl-terminal fragments of TSHα and TSHβ , no significant recombination of these with each other or with native TSHα or TSHβ occurred; however, both TSHα and LHα , after hydrolysis, recombined with native LHβ although the recombinant molecules were inactive (17). Carboxymethylation

of the methionine residues of bLHα and bLHβ did not modify their ability to recombine or their immunological activity, however the recombinant hormone exhibited $< 5\%$ of the biological activity of intact bLH (18). Recombination of one modified subunit with one intact subunit regenerated 20 - 30% biological activity (18).

The Presence of Free Subunits under Various Physiological Conditions

The presence of free subunits has been reported under certain physiological conditons. Prentice and Ryan (21) have reported an excess of free α-subunit in the pituitaries, sera and urine of post-menopausal women. Edmonds et al. (33) have also reported free α-subunit in the sera of post-menopausal women as well as in the sera of normal subjects. Serum and urine samples from pregnant women contained free HCGα and HCGβ ; however, the levels of the free HCGα were much greater than those of the β-subunit (40). Vaitukaitis (44) has demonstrated the presence of free HCGα in human placental tissue from normal term pregnancies. Studies on the metabolic clearance rates of the α and β subunits of hTSH indicated that the free subunits detected in serum are probably of pituitary origin and not a by-product from the degradation of intact glycoprotein hormones (38).

Presence of Free Subunits under Certain Pathological Condtions

The presence of free circulating subunits has been well documented under various disease conditions. Elevated free α-subunit has been found in both serum from patients with oat cell carcinoma of the lung and from patients with chronic renal failure (36). Human pituitary extracts from patients with atrophic asymptomatic thyroiditis contained free TSHα and TSHβ which were separable from intact TSH after gel filtration on Sephadex G-100 (31). Patients with pituitary adenomas were found to have elevated serum free α -subunit levels in addition to a decreased response to TRH and LHRH implying a relative autonomy of the pituitary tumor secreting free α-subunit (32). Serum levels of 30,000 ng/ml of HCGα were detected in an isolated case of a gastric carcinoma (42); whereas in one case of pancreatic adenosquamous carcinoma, serum levels of 300 ng/ml HCGβ were observed (43). In addition to the reports of elevated serum concentrations of subunits as a result of abnormal pathological conditions, one *in vitro* study reported that the β-subunit of bTSH has exopthalmogenic activity (24).

The Effect of Releasing Factors on the Serum Levels of Free Subunits

Benveniste et al. (22) reported that free α-subunit levels in human serum increased within minutes after

administration of LHRH but that these levels return to normal within 1 hour. In the study of 20 hypothyroid patients, elevated levels of hTSHβ and free α-subunit were detected after TRH administration (25).

Increases of serum levels of hTSHβ in 11 hypothyroid patients after TRH administration parallel the increase of serum hTSH (29). Hagen and McNeilly (34) have reported that the rise of free α -subunit in response to TRH and LHRH administration preceeded the rise of the intact hormone.

Free α Subunit in Culture

Franchimont et al. (40) reported that culture of human placental tissue produced large amounts of free α-subunit in conjunction with very small or non-detectable levels of β-subunit. In addition, culture of cells from a gastric carcinoid tumor resulted in a cell line which synthesizes mainly ectopic HCGα subunit (23).

Studies of Glycoprotein Hormones and their Subunits in Maternal Serum, Fetal Serum and Fetal Pituitary Glands at Various Stages of Pregnancy

To date, studies in the area of glycoprotein hormones and their subunits in maternal serum, fetal serum and fetal pituitary glands at various stages of pregnancy, have been carried out mostly in humans. Hagen and McNeilly(19,20)

have reported that at the time of delivery, circulating levels of FSH were similar in maternal and fetal sera; whereas the levels of HCGα, HCGβ, LHα and LHβ were significantly higher in the maternal serum. There was no correlation between serum levels of these hormones in maternal and fetal circulations. Studies with pituitary glands from human fetuses of between 9.5-32 weeks gestational age showed that all the pituitary glands contained free α-subunit and LH (35); however the ratio of free α-subunit:LH was higher in pituitary glands from younger fetuses (9.5-16 weeks gestational age). In the same study (35), no free LHβ or FSHβ were detected, and no difference in the fetal pituitary levels of LH were observed owing to the sex of the fetus. In one study utilizing bovine fetal pituitary glands, Oxender et al. (26) reported that LH levels (ng/mg pituitary tissue) increased gradually between days 90, 180 and 260 of gestation and that no difference was found between LH levels in fetal pituitary glands from males and females. In contrast, in human fetal pituitary glands, Kaplan et al. (37,41) reported significantly higher LH levels in female fetuses over male fetuses after the 14th week of gestation. The levels of FSH were also reported to be higher in human fetal pituitary glands from females over males after the 14th week of fetal age (37,41); and the same was true for serum levels of FSH (30,37,41). This difference

was attributed to the increase of testosterone levels occuring in the serum of 11 to 17 week old males fetuses as a result of differentiations of the genital tract and the development of a functional hypothalamic pituitary-gonadal feedback system (30,37,39).

In a study of TSH levels in human fetal pituitary glands obtained from 40 fetuses between 8-32 weeks of gestation, Fukuchi et al. (27) reported that the TSH content in the fetal pituitary/gm fetal body weight was maximal at 12-17 weeks. In studies on fetal calves, Thomas et al. (28) reported that serum TSH levels varied greatly until a week before delivery and then gradually fell to near zero on the day before parturition.

Specific Aims and Objectives

In the literature, there have been some scattered reports on the levels of LH, FSH or TSH and the α and β subunits of these hormones in fetal pituitary glands. Due to the lack of techniques in any one laboratory at this time to measure all of these hormones and subunits, previous reports have been focused on monitoring levels of only one or two of the components. In addition, most of these studies were carried out in humans with very limited sample size of fetal pituitary glands at different stages of gestation. Almost no information is available with respect to this aspect of studies in any other species.

In this laboratory, highly purified bLH, TSH and their α and β subunits, and recently, purified bFSH and its subunits were all available. In addition, radioreceptor assays for bLH and bFSH as well as radioimmunoassays for bLH, bTSH and their α and β subunits were also available.

With the availability of these highly purified preparations of hormones and assay systems, the present studies were set forth to examine the correlation between LH, FSH, TSH and their α and β subunits in fetal pituitary glands in the bovine species.

METHODS AND MATERIALS

Reference Hormones

Purified bLH (potency: 2.0 x NIH-LH-S-1), bTSH (potency: 30 i.u./mg) and the α and β subunits of bLH and bTSH were gifts from Dr. J.G. Pierce, UCLA, Los Angeles, Ca. Purified bFSH (potency: 164 x NIH-FSH-S-1) was prepared in our laboratory (Cheng, K.W. Biochem. J. 159: 651, 1976). The preparation of the α and β subunits of bFSH was also accomplished in our laboratory (Cheng, K.W. Biochem. J., in press). Purified oPRL (NIH-P-S-12), bGH (NIH-GH-B18) and rTSH (potency: 35 i.u./mg) were from the National Institutes of Health, Bethesda, Md., U.S.A. Porcine insulin potency: 24.2 i.u/mg (Lot #1119) was obtained from Connaught Laboratories, Toronto, Ont., Canada.

Chemicals

BSA (fraction V), bovine -γ- globulin, and chloramine-T were from the Sigma Chemical Co., St. Louis, Mo., U.S.A Lactoperoxidase was from Calbiochem, San Diego, Ca., U.S.A. $Na^{125}I$ (carrier-free) was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. Sephadex G-100 was from Pharmacia, Dorval, Que., Canada. All other reagents and chemicals were reagent grade from either Fisher Scientific Co., Fairlawn, N.J., U.S.A. or J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.

Antisera

Specific rabbit antisera against bFSH, bFSHα and bFSHβ were raised in our laboratory. Specific rabbit antisera against bTSH, bTSHβ and bLHβ were gifts from Dr. J.G. Pierce, UCLA, Los Angeles, Ca., U.S.A.

Experimental Animals and Samples

Six ten-week old New Zealand white rabbits used for immunization were obtained from Reimer's Fur Ranches, St. Agatha, Ont., Canada. Fresh bovine fetal pituitary glands, porcine thyroid glands and other animal tissues were from Burns Meat Ltd., Winnipeg, Manitoba, Canada.

Protein Measurements

Protein elution patterns of pituitary extracts after gel filtration on Sephadex G-100, were monitored by measuring absorbance at 278 nm of the eluant fractions on a Beckman Model 25 Spectrophotometer.

To estimate the protein concentration of membrane preparations, a slight modification of the method of Lowry et al. (69) was used. Membrane preparations were diluted 1:2, 1:5, 1:10 and 1:20 in distilled water. Samples of 100 μl of each sample were pipetted into 15 x 150 nm pyrex test tubes followed by the addition of 300 μl of 1N NaOH. The samples were then boiled for 30 minutes to solubilize the membrane protein. A stock solution of 100 ml of 2% Na_2CO_3, 1 ml of 1% $CuSO_4$ and 1 ml of 2% Na/K tartrate was freshly prepared and 4 ml of this solution was then added to the reaction tubes containing the protein solution. After 10 minutes, 0.5 ml of 1N phenol solution was added to each tube followed by immediate mixing on a vortex-genie. After 30 minutes, absorbance of the samples was read at 660 nm on the spectrophotometer. BSA, prepared at concentrations of 2,5,10,15 and 20 mg/100 ml distilled water, and undergoing the same treatment as the membrane preparations, was used as standards.

Iodinations

Bovine LH, bTSH the α subunit of bFSH and the β subunits of bLH, bTSH and bFSH were prepared by the chloramine T method of Greenwood, Hunter and Glover (45). All reactions were at room temperature. $Na^{125}I$ (0.5 mCi) in 25 μl of 0.5 M PO_4 buffer, pH 7.0, was pipetted into a 12 x 75 mm disposable glass culture tube containing 5 μg hormone in 25 μl of 0.1 M NH_4HCO_3 buffer. Chloramine-T (50 μg) in 25 μl of 0.05 M PO_4 buffer, pH 7.5 for use as the oxidizing agent, was pipetted into the culture tube and allowed to react for 30 seconds. This was followed by the addition of 125 μg of sodium meta bisulfate in 50 μl of 0.05 M PO_4 buffer, pH 7.5 in order to convert ^{125}iodine into ^{125}iodide, thus preventing any subsequent iodination of the Sephadex G-100 gel bed. Potassium iodide (750 μg) in 75 μl of 0.05 M PO_4 buffer, pH 7.5 was then added to the reaction mixture to dilute the residual iodide. The reaction mixture was further diluted by the addition of 2.3 ml PBS, pH 7.4 bringing the total volume of the reaction mixture to 2.5 ml. The chloramine-T, sodium meta-bisulfate and potassium iodide were freshly prepared and dissolved immediately before use.

Bovine FSH was iodinated by the lactoperoxidase method (70) with slight modification according to the method of Cheng (50). All reactions were at room temperature. $Na^{125}I$ (0.5 mCi) in 25 μl of 0.5 M PO_4 buffer, pH 7.0, was added to a 12 x 75 mm disposable glass culture tube containing 5 μg bFSH in 25 μl of 0.1 M NH_4HCO_3 buffer and 25 μl of 0.05 M PO_4 buffer, pH 7.0. Lactoperoxidase (5 μg), the oxidizing

agent, in 5 μl of 0.05 M PO_4 buffer, pH 7.0, was then added to the reaction tube. The lactoperoxidase was activated by two 10 μl additions of hydrogenperoxide at 1:15,000 dilutions with distilled water of the original 30% solution at 5 minute intervals. At the end of the second 5 minute period, 2.4 ml of 0.025 M Tris-HCl buffer, pH 7.4 was added to the reaction tube to stop the reaction, yielding a final volume of 2.5 ml.

Unreacted iodide and damaged hormones were separated from the iodinated hormone by gel filtration on a 2 x 45 cm column of Sephadex G-100 using PBS, pH 7.4, as the eluting buffer for the chloramine-T method and 0.025 M Tris-HCl, pH 7.4 as the eluting buffer for the lactoperoxidase method. The column was pretreated with 2 ml of 2.5% BSA in the appropriate buffer (W/V) in order to minimize the loss of iodinated proteins adhering to the Sephadex.

Fractions of 3 - 4 ml were collected in 15 x 50 mm glass pyrex test tubes. The peaks of radioactivity were monitored with a Chicago Nuclear geiger counter.

For bLH, bFSH and bTSH, the peak of radioactivity containing the iodinated hormone was determined by the specific binding test as described in the Specific Binding of radioreceptor assays section of Methods and Materials.

After iodination of the α subunit of bFSH or the β subunits of bLH, bFSH or bTSH, the peak of radioactivity of the labelled hormone was determined by its ability to bind to an excess amount of the appropriate antiserum at a 1:1000

dilution in PBS, pH 7.4, containing 0.1% BSA. One hundred microliters of ^{125}I-labelled hormone (25,000 CPM) in PBS, pH 7.4, containing 0.1% BSA was incubated with both 100 μl antiserum and 100 μl of PBS. Separation of antibody - bound and free ^{125}I-labelled hormone was accomplished as described in the Radioimmunoassay Technique section of Methods and Materials.

Radioreceptor Assays for bLH, bFSH and bTSH

Radio-receptor assays for bLH and bFSH, using a particulate receptor preparations from porcine testes, were specific for the ligand being used. The procedure for the preparation of the particulate membrane fraction was identical to the published method for bovine testes (50). A radio-receptor assay for bTSH was developed using a crude membrane preparation from porcine thyroid glands. Fresh porcine thyroid glands were trimmed of all extraneous tissue washed with normal saline, cut into small pieces, and homogenized in 0.3 M sucorse utilizing a Brinkman Polytran homogenizer at high speed in a ratio of 1 gm wet weight tissue/ 5 ml sucrose. The homogenate was filtered through four layers of cheese cloth followed by centrifugation of the filtrate at 10,000 x g for 30 minutes on a Beckman Model J-21B centrifuge. The supernatant was collected and centrifuged at 100,000 x g for 60 minutes in a Beckman Model L5-65 Ultra-centrifuge. The supernatant was discarded and the pellet was resuspended in 0.025 M Tris-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$, in a ratio of 1 ml buffer/1 gm wet weight thyroid gland tissue. The membrane bound receptor preparation was then either used in the radioreceptor assay for bTSH or was stored at -20° where it could be kept for up to 6 months

without any significant loss of receptor binding activity.

Specific Binding of Radioreceptor Assays

Radio-actively labelled bLH, bFSH and bTSH were tested for their ability to bind specifically to their appropriate membrane receptor preparations. In the specific binding test for bLH, ^{125}I-bLH was diluted to a final concentration of approximately 50,000 CPM/100 μl in Tris-HCl buffer, pH 7.2, containing 10 mM Mg Cl_2 and 0.1% BSA. This tracer (100 μl) was incubated in duplicate in 12 x 75 mm disposable glass culture tubes with either 100 μl of bLH at a concentration of 1000ng/ml or 100 μl of the assay buffer alone. Particulate receptor (100 μl) was added to each tube at the appropriate dilution followed by a further addition of 200 μl of the assay buffer. After 16 hours incubation at 25°, 3 ml of the ice-cold assay buffer was added to each tube to stop the reaction. The pellet of the membrane bound tracer was obtained by centrifuging at 1,500 x g for 30 minutes in an IEC-PR-6000 centrifuge, followed by decanting the supernatant and drying the edges of the assay tubes with kim-wipes. Radio-activity was counted in an automatic gamma counter.

The percentage of specific binding was calculated as the CPM that was specifically displaced by 1000 ng/ml cold hormone x 100 divided by the total CPM in the reaction tube.

$$\% \text{ Specific Binding} = \frac{(\text{Bound CPM} - \text{non Displacable CPM}) \times 100}{\text{Total CPM}}$$

The procedure to test for the specific binding of ^{125}I-bFSH was identical to that outlined for ^{125}I-bLH. The only

difference in the specific binding test for ^{125}I-bTSH, was that the final 200 μl of Tris-HCl buffer, pH 7.2, 10 mM Mg Cl_2, 0.1% BSA, was not added into the tubes.

Suitable fractions of ^{125}I-labelled hormones were stored at -20° until required.

Immunization Procedures

Specific rabbit antisera against bFSH, bFSH α and bFSH β were raised in this laboratory using two rabbits to generate the antiserum against each antigen. For immunization, the hormones were dissolved in 0.5 ml of normal saline. This was then drawn into a 2ml Plastipak syringe with a 16 gauge needle. An equal volume of Fruend's complete adjuvant (0.5 ml) was drawn into another identical syringe. The two syringes were connected by a 1/2 " piece of rubber tubing and the adjuvant and hormone were transferred back and forth between the two syringes until a white homogeneous mixture was obtained. To prepare the rabbits for immunization, their backs were shaved and cleaned with a small amount of ethanol to prevent infection. The rabbits were injected intradermally, the first injection containing 200 μg of appropriate hormone/rabbit. This was followed by an additional injection of 200 μg hormone/rabbit two weeks later. Subsequent injections were with approximately 50 μg hormone/rabbit every second week for the next sixteen weeks. The 1 ml volume used for injection allowed for approximately 20 intradermal injection sites/rabbit. Rabbits were bled by applying a small amount of toluene to the base of the peripheral ear vein in order

to dilate the vein, nicking the vein with a scalpel blade, and collecting the blood in 40 ml conical glass pyrex centrifuge tubes. An average of 30 ml blood was collected from each rabbit/bleeding. The first bleeding was performed 10 days after the third immunization. Subsequent bleedings were 10 days after each booster.

After the blood was collected, the centrifuge tubes containing the blood were placed in ice for 30 minutes in order to allow the blood to coagulate. They were then centrifuged at 1500 x g for 30 minutes at 4° in an IEC PR-6000 centrifuge to separate the serum from the red blood cells. The serum was then carefully pipetted into 10 ml glass vials and stored at -20° until use.

Radioimmunoassay Technique

The radioimmunoassays utilized 100 μl of the antibody (diluted appropriately in PBS, pH 7.4, containing 0.1% BSA) added in duplicate to 12 x 75 mm disposable glass culture tubes containing 100 μl sample and 100 μl ^{125}I-labelled hormone (25,000 CPM) in PBS, pH 7.4, containing .1% BSA. The tubes were incubated for 24 hours at 25°. Separation of antibody-bound and free ^{125}I-labelled hormone was achieved by the addition of 0.2 ml of PBS buffer, 0.5 ml of 2% bovine - γ-globulin in PBS buffer and 1 ml of 22% polyethylene glycol in the same buffer to each tube followed immediately by vortexing.

The tubes were centrifuged at 1,500 x g at 4° for 30 minutes in an IEC-PR-6000 centrifuge and the supernatant

was aspirated. The radioactivity in the precipitate was counted in a Searle automatic gamma counter.

Preparation of Pituitary Extracts

Excised bovine fetal pituitary glands were placed in 12 x 75 mm disposable glass culture tubes and kept on ice until use (2 - 3 hrs.). The pituitary glands were then weighed and homogenized by hand in cold Tris-HCl buffer, pH 7.2, at concentrations of 5, 10, 20, 25, 50 or 100 mg pituitary gland tissue/ml buffer in order to yield a final homogenate volume of between 1 and 8 mls. The homogenate was centrifuged at 10,000 x g for 30 minutes in a Beckman Model J-21B centrifuge. The supernatant was then collected and stored at -20° until use. In total, 134 fetal pituitary glands obtained from fetuses at different stages of gestation, were prepared in this manner.

For characterization of the RRA for bTSH, pituitary extracts from cow, dog, rabbit, rat, guinea pig, turkey and chicken pituitary glands were prepared at a concentration of 10 mg pituitary tissue/ml buffer in an identical manner.

Column Chromatography

Pituitary extracts (1 ml in volume) were chromatographed on a 1 x 90 cm or 2 x 110 cm column of Sephadex G-100 equilibrated in 0.5% NH_4HCO_3 buffer at 4°. Fractions containing 2.2 ml (50 drops) and 1.15 ml (25 drops) respectively, were collected from the small and large columns in 12 x 75 mm disposable glass culture tubes on a Buchler Instruments Fractomette 200 automatic fraction collector.

The void volume of the columns was determined by chromatographing 1 ml of Blue Dextran at a concentration of 10 mg/ml in 0.5% NH_4HCO_3 buffer, and monitoring the eluant fractions for the appearance of the Blue Dextran at a wave length of 660 nm in a Beckman Instruments, Model 25 spectrophotometer. The salt peak was determined by the chromatography of free Na ^{125}I and by counting the eluant fractions on an automatic gamma counter.

The protein elution patterns of the pituitary extracts were determined by monitoring the eluant fractions at an absorbance of 278 nm.

The peak of intact hormone or α and β subunit activities was determined by assaying the eluant fractions with the specific RRAs or RIAs. Fractions containing hormone activities were pooled, lyophilized, and stored at 4° until required for further studies.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Davis (71). A 7.5% (w/v) acrylamide gel was used at pH 8.9. The electrode buffer used was 0.012 M sodium glycinate, pH 9.5. Samples were dissolved in 50 μl electrode buffer, followed by the addition of 1 drop of glycerol to increase the density of the sample, and 10 μl of 0.005% bromophenol blue for marking. A current of 2 m Amp/gel was applied until the ion front in the gel travelled a distance of 6 cm from the origin. The gels were then either stained for protein with 1% amido black

in 7% acetic acid followed by destaining with 7% acetic acid, or were segmented in 5 mm slices. Each slice was further cut into several smaller pieces and eluted in a 12 x 75 mm disposable glass culture tube for 24 hrs with 1 ml of .5% NH_4HCO_3 buffer. The eluates were then pipetted into glass tubes and the gel segments were further washed for an additional 24 hours in 0.5 ml of the same buffer. The eluates of the same gel segments were then pooled, lyophilized, and redissolved in 0.5 ml of 0.025 M Tris-HCl buffer, pH 7.2, containing 0.1% BSA. The samples were then further appropriately diluted in the same buffer for assaying of activity by the appropriate RRA or RIA.

Recombination Studies

Bovine FSH, bFSHα, bFSHβ and the lyophilized fraction of free α-subunit activity from fetal pituitary extracts after Sephadex G-100 column chromatography were dissolved in 0.012 M sodium glycinate buffer, pH 9.5, at a concentration of 1 μg activity/ml buffer. Various α-subunit preparations (10 μl) were incubated with 10 μl bFSHβ in a nitrogen atmosphere for 16 hours at 37°. The reaction was stopped by diluting the incubation mixture to 1 ml with ice-cold 0.025 M Tris-HCl buffer, pH 7.2, containing 0.1% BSA. The samples were then appropriately diluted in the same buffer and immediately assayed by the RRA for bFSH in order to determine the amount of intact bFSH activity regenerated. Samples containing only bFSH, bFSHα or bFSHβ, treated in the same manner, were used as standards.

Calculation of α and β Subunit Activity

The radioreceptor assays for bLH, bFSH and bTSH used in these studies have been shown to be specific for these hormones, whereas in the radioimmunoassays for the various subunits, the intact glycoprotein hormones cross react significantly at levels ranging from 10% to 30%.

Extracts from bovine fetal pituitary glands were assayed by the radioreceptor assays for bLH, bFSH and bTSH to determine the exact amount of these hormones present.

The same bovine fetal pituitary extracts were assayed by the RIA for bFSHα to determine the total amount of immunoreactive α-subunit activity present.

In the RIA for bFSHα, it was found that bLH, bFSH and bTSH all cross reacted at a level of 10%.

Table 1 shows the method of calculation used to determine the absolute amount of α-subunit activity present in bovine fetal pituitary gland extracts.

The values of bLH, bFSH and bTSH found to be present in the pituitary extract, as determined by the RRA's, were individually multiplied by 10% in order to determine the amount of cross reactivity caused by this individual hormone in the RIA for bFSHα ((X) x 10%, (Y) x 10% and (Z) x 10%). The figures obtained for the cross reactivity due to the presence of bLH, bFSH and bTSH were then added to give a value showing the total cross reactivity in the RIA for bFSHα due to the presence of the three intact glycoprotein hormones ((X) x 10%) + ((Y) x 10%) + ((Z) x 10%) = total cross reactivity in the bFSHα RIA due to the presence of intact glycoprotein hormones).

TABLE 1 : Calculation of absolute amounts of free α-subunit in pituitary extracts.

Intact Hormone	Amount of Hormone as determined by RRA	Cross-reactivity in the α-subunit RIA of individual glycoprotein hormones	Total cross-reactivity in the α-subunit RIA due to intact hormones
bLH	X	X x 10%	{X x 10%}
bFSH	Y	Y x 10%	{Y x 10%}
bTSH	Z	Z x 10%	{Z x 10%}

Formula used to obtain the absolute amount of α-subunit in pituitary extracts.

[Total amount of immunoreactive α-subunit in pituitary extract as determined by RIA.] - [Total cross-reactivity in the α-subunit RIA due to intact hormones] = [Absolute amount of α-subunit in pituitary extract.]

This value was then subtracted from the amount of total immunoreactive α-subunit activity, as determined by the RIA for bFSHα, resulting in an exact value showing the absolute amount of immunoreactive α-subunit present in the bovine fetal pituitary extract.

The absolute amounts of bLHβ, bFSHβ and bTSHβ were determined in the same manner. The percentage of cross-reactivity of the intact hormones in the different RIA's for the various subunits, are summarized under Characterization of Radioimmunoassays in the Results section of this thesis.

Determination of Fetal Age and Sex

The age of every individual fetus was determined by its crownrump length. The relationship between the fetal crown-rump length (in inches), and the fetal age (in months) is shown in Table 2 (72). The sex of each individual fetus was determined by its external sexual features, which can be clearly distinguished after a gestation period of 2½ months (68).

Plotting of Data

The data obtained from the radioreceptor assays for the intact glycoprotein hormones and by the radioimmunoassays for the α-subunit was plotted in terms of both ng hormone/mg wet weight pituitary tissue and μg hormone/pituitary gland. The ratios of α-subunit to individual glycoprotein hormones as well as the ratio of α-subunit to the total glycoprotein hormone content per pituitary gland were also plotted.

TABLE 2 : Fetal body-length (Crown-Rump) in relation to various stages of pregnancy.

Pregnancy (months)	Fetal Crown-Rump length (inches)
1	1/3
2	2 1/2
3	6
4	11
5	16
6	21
7	28
8	32
9	36

Grouping of Samples

In order to see if there were any significant changes in hormone levels in fetal pituitary glands related to the age or sex of the fetus, results of the hormone content obtained from the assay of fetal pituitary extracts were arbitrarily divided into eight groups according to the age and sex of the fetus.

Groups 1 - 4 were results from the assay of pituitary extracts from male fetuses of ages less than 3 3/4 months, 3 3/4 - 5 months, 5 - 6 1/4 months and greater than 6 1/4 months. Groups 5 - 8 were results from the assay of fetal pituitary extracts from female fetuses grouped into the same age divisions as the males.

Statistical Analysis

In order to determine if there was any difference in the level of each hormone between groups 1 - 8, a two way analysis of variance was performed on these groups utilizing Duncan's new multiple range test (73). This test is used to find significant differences between groups containing different n values. A p value of $<.05$ was interpreted to be a statistically significant difference.

RESULTS

Development of a Radioreceptor Assay for bTSH

Because of the cross-reactivity of bTSH in the RIA for bTSHβ, a specific assay system for measuring bTSH was required. For this reason, a specific RRA for bTSH was developed, and the optimal conditions for this assay was characterized.

Preparation of Plasma Membranes from Porcine Thyroid Gland

A crude membrane preparation from porcine thyroid glands was used as the target receptor preparation for ^{125}I-bTSH. Fresh porcine thyroid glands were trimmed off all extraneous tissue, washed with normal saline, cut into small pieces, and homogenized in 0.3M sucrose utilizing a Brinkman Polytron homogenizer at high speed in a ratio of 1 gm wet weight tissue per 5 ml sucrose. The homogenate was filtered through four layers of cheesecloth, followed by centrifugation of the filtrate at 10,000 x g for 30 minutes on a Beckman Model J-21B centrifuge. The supernatant was collected and centrifuged at 100,000 x g for 60 minutes in a Beckman Model L5-65 Ultracentrifuge. The supernantant was discarded and the pellet was resuspended in 0.025M Tris-HCl buffer, pH 7.2, containing 10mM $MgCl_2$, in a ratio of 1 ml buffer per 1 gm wet weight thyroid gland tissue. The membrane bound receptor preparation was then either used in the radioreceptor assay for bTSH or was stored at -20° where it could be kept for up to 6 months

without any significant loss of receptor binding activity.

Optimal pH

To determine the optimal pH for the RRA, 0.025M Tris buffer, containing 10mM $MgCl_2$ and 0.1% BSA was titrated to pH's 8, 7.7, 7.4, 7.1, 6.8 and 6.5 with 1 N HCl. Porcine thyroid membranes, bTSH (1000 ng/ml) and ^{125}I-bTSH (approximately 50,000 CPM) were individually prepared in the buffers at each pH. Two sets of duplicate tubes, with and without unlabelled hormone, were prepared for each pH to obtain the specific binding of ^{125}I-bTSH. As shown in figure 1, the specific binding for ^{125}I-bTSH was 5% at pH 6.5, and this increased to 6% binding at pH 7, but decreased to 4% at pH 8.

Effects of Salts

In order to examine the effects of various salts on the binding of ^{125}I-bTSH, $MgCl_2$, $CaCl_2$, KCl and NaCl were prepared at 3M concentrations in Tris-HCl buffer, pH 7.2 containing 0.1% BSA. These were then diluted with the same buffer to yield solutions containing 1.5, 0.75, 0.3, 0.15, 0.075, 0.03, 0.015, and 0.0075 M of salt. One hundred microliters of each solution was then incubated in duplicate with 100 μl membrane receptor in 0.025M Tris-HCl buffer, pH 7.2 and 100 μl of ^{125}I-bTSH (diluted to 50,000 CPM in 0.025 M Tris-HCl buffer, pH 7.2, containing 0.1% BSA) yielding final salt concentrations of 1, 0.5, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.0025M. As can be

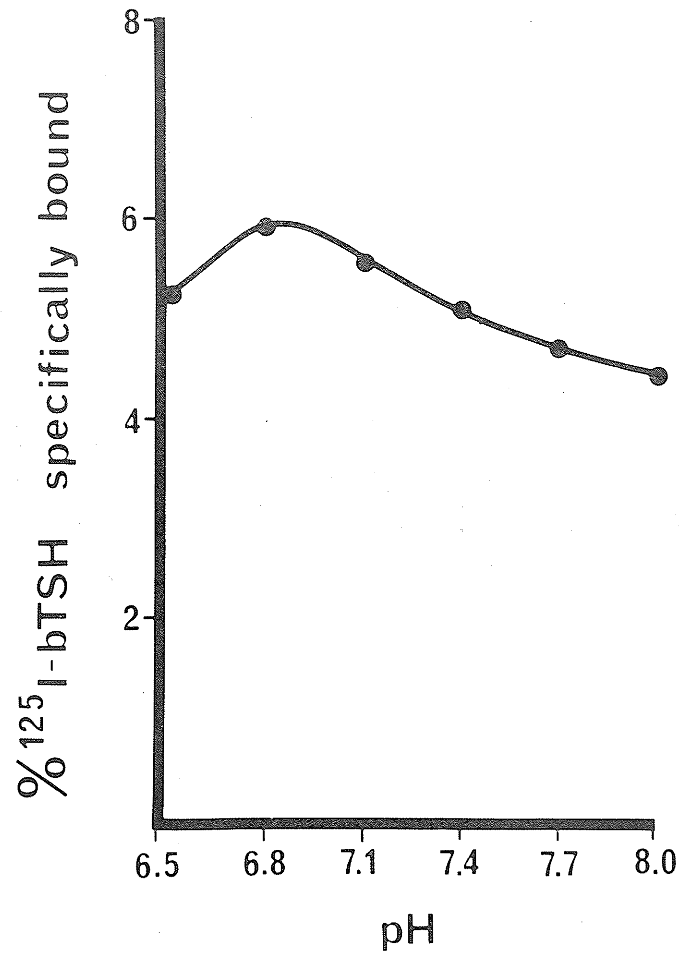

Figure 1 : The effect of pH on the specific binding of ^{125}I-bTSH to porcine thyroid membranes.

seen in figure 2, ionic concentrations greater than 0.01M for every salt tested, inhibited ^{125}I-bTSH binding to porcine thyroid membranes. Thereafter, RRA buffer was prepared containing 10mM $MgCl_2$.

Effects of Increasing Amounts of Membrane Protein

Membrane preparations from porcine thyroid glands, suspended in 0.025M Tris-HCl buffer, pH 7.2, containing 10mM $MgCl_2$, were found to contain approximately 300 μg protein/100 μl buffer as determined by the method of Lowry et.al. (69). The membrane preparations were centrifuged at 100,000 x g for 60 minutes in a Beckman Model L5-65 Ultracentrifuge and the membrane pellet was resuspended in 0.025M Tris-HCl buffer, pH 7.2, containing 10mM $MgCl_2$ at a concentration of 1200 μg membrane protein/ 100 μl buffer. The membranes were further diluted in the same buffer to yeild membrane protein concentration of 900, 600, 300 and 150 μg/100 μl buffer. Each dilution was then tested for its ability to bind ^{125}I-bTSH.

Figure 3 shows that there was a direct relationship between the specific binding of ^{125}I-bTSH and the amount of membrane protein for the range tested. For assay purposes, 300-600 μg membrane protein/tube was used depending on the specific binding capacity of the particular preparations of thyroid membranes.

Time and Temperature Effects

In order to determine the optimal incubations conditions for the RRA for bTSH, the specific binding of

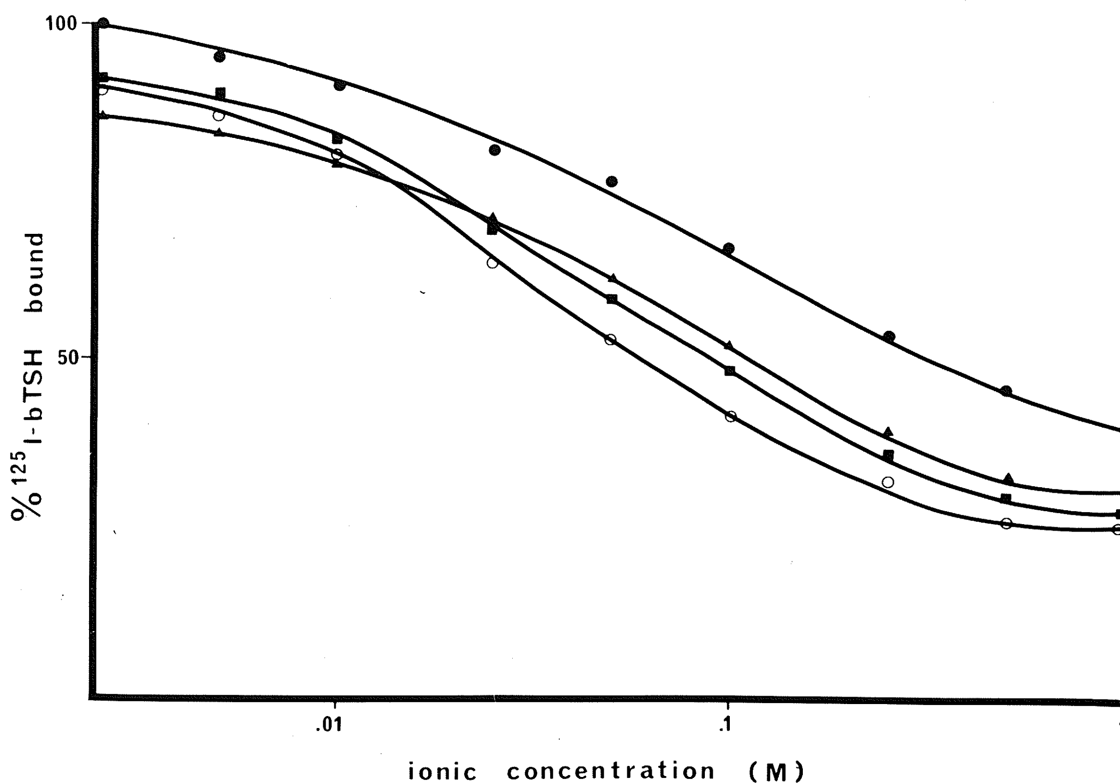

Figure 2 : Effect of different ionic concentrations of various salts on the binding of ^{125}I-bTSH to porcine thyroid membranes.

●—● = KCl; ■—■ = $MgCl_2$; ○—○ = $CaCl_2$; ▲—▲ = NaCl.

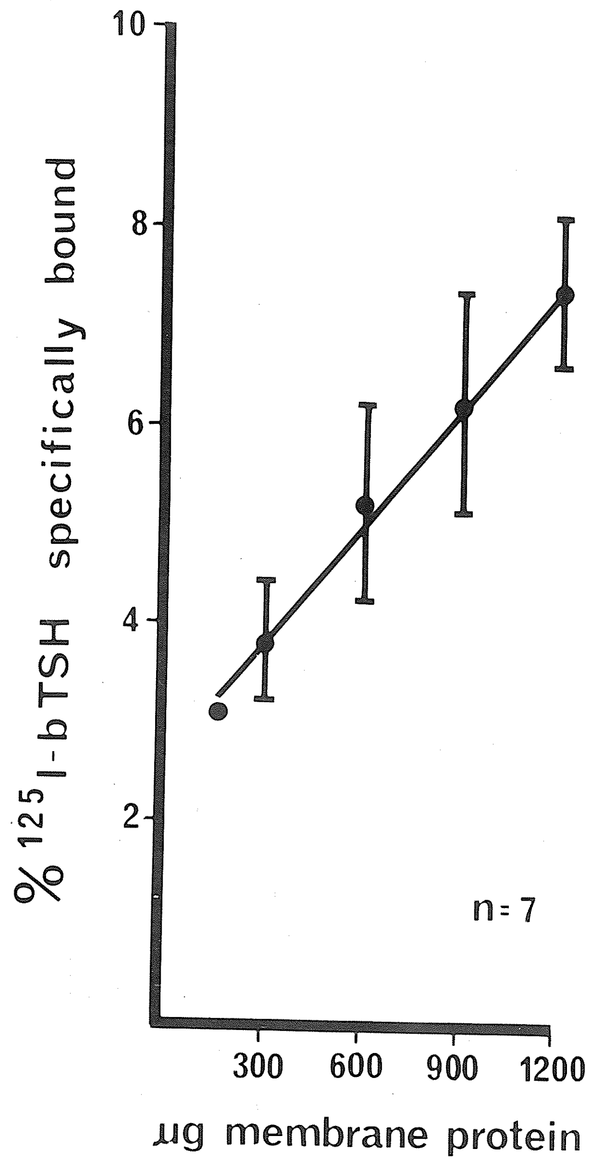

Figure 3 : Effect of increasing membrane protein concentrations on the specific binding of ^{125}I-bTSH to porcine thyroid membranes.

^{125}I-bTSH to porcine thyroid membranes was studied at various time intervals and temperatures. Duplicate assay tubes containing 100 µl of particulate receptor (300 µg membrane protein) and 100 µl of ^{125}I-bTSH (approximately 50,000 CPM) in the presence and absence of 100 µl bTSH (1000 ng/ml) were incubated at 4°, 25° and 37° at time intervals ranging from 15 minutes to 24 hours.

Maximal specific binding (approximately 4%) was observed after both 1 and 4 hour incubations at 37° and 25°, respectively (figure 4). Under both conditions, specific binding decreased after reaching its maximal level due to an increase of non-specific binding with time. At 4°, specific binding increased linearly and reached a level of 2% after 24 hours.

For assay purposes, a 37° incubation for 1 hour was routinely used.

Characterization of the Radioreceptor Assay for bTSH

Sensitivity and Specificity

In order to determine the sensitivity of the assay, 100 µl of membrane receptor (300 µg protein) and 100 µl of ^{125}I-bTSH (approximately 50,000 CPM) were incubated with 100 µl of varying concentrations of TSH (1-100 ng/ml). Bovine TSH and rTSH of similar biological potencies displaced ^{125}I-bTSH from the membrane receptor at concentrations of 20 ng/ml. Maximum displacement occurred at a

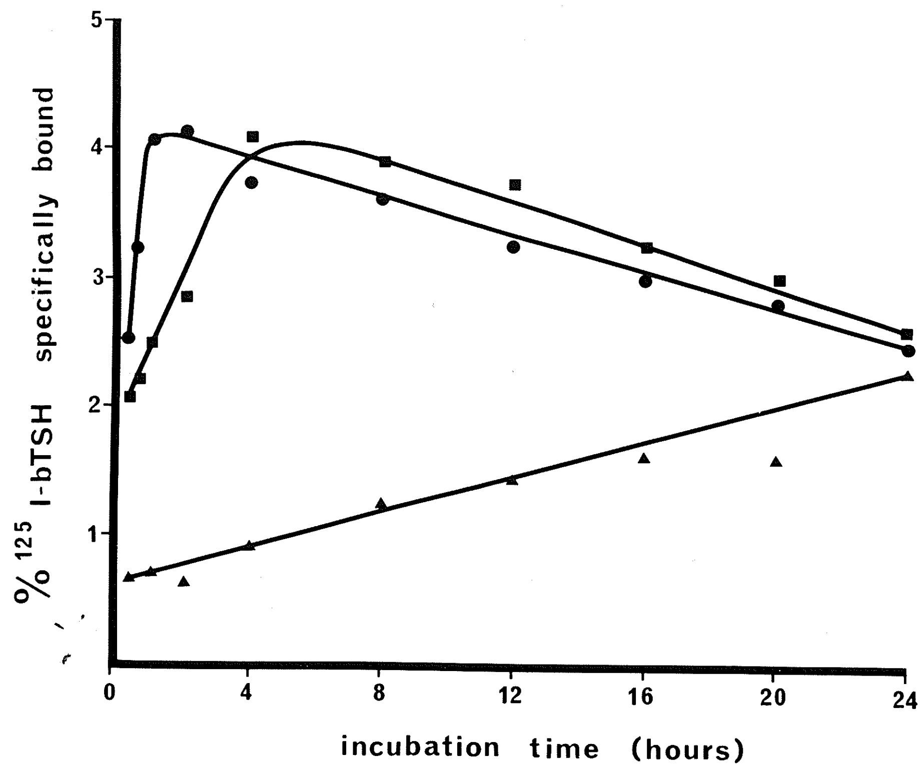

Figure 4 : Effects of time and temperature on the specific binding of ^{125}I-bTSH to porcine thyroid membranes. ▲—▲ = incubation at 4°; ■—■ = incubation at 25°; ●—● = incubation at 37°.

concentration of 300 ng/ml. The intra-assay varience was found to be less than 10%.

The specificity of the RRA for bTSH was determined by incubating the membrane receptor with various concentrations of other protein and polypeptide hormones. As shown in figure 5, bLH, bFSH, bTSHα, bTSHβ, bGH, oPRL and and pInsulin, up to concentrations of 10,000 ng/ml, did not displace ^{125}I-bTSH from the membrane receptor.

Comparison of RRA and RIA results

In order to test the accuracy of the radioreceptor assay, extracts from bovine pituitary glands were assayed by RRA and the results were compared to values obtained by assaying the same samples by an established RIA for bTSH.

Eleven bovine pituitary glands were individually cut into small pieces and homogenized by hand in 0.025M Tris-HCl buffer, pH 7.2, in a ratio of 100 mg pituitary tissue/ml buffer. The homogenates were centrifuged at 10,000 x g for 30 minutes in a Beckman Model J-21B centrifuge. The supernatants were collected, and appropriately diluted in 0.025M Tris-HCl buffer prior to assay.

The content of bTSH in each pituitary extract as measured by both RRA and RIA was similar (Table 3). The mean and standard deviation of TSH activity in the 11 bovine pituitaries was also similar by both RRA and RIA (120.29 $\pm$ 31.18 ng/mg pituitary tissue and 111.39 $\pm$ 19.81

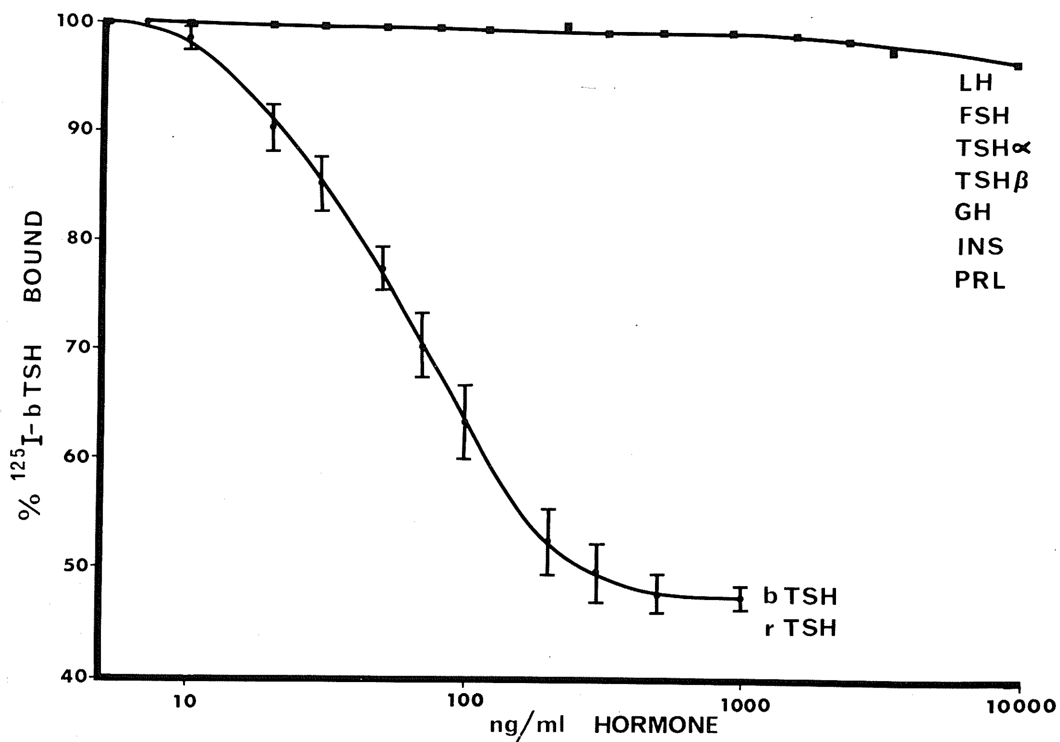

Figure 5 : Standard curves for bTSH and other protein and polypeptide hormones in the RRA using porcine thyroid membranes showing the sensitivity and specificity of the RRA system. ●—● = bTSH, rTSH. ■—■ = bLH, bFSH, bTSHα, bTSHβ, bGH, oPRL, pInsulin.

TABLE 3 : Comparison of values for activity in bovine pituitary extracts by TSH RRA to an established bTSH RIA.
Values for TSH activity in an individual samples were averages of duplicate measurements. The means and standard deviations of the groups were similar by both RRA and RIA as indicated by the RRA/RIA ratio being close to 1.

Bovine Pituitary Extract	bTSH Activity ng/mg Tissue		
	RRA	RIA	RRA/RIA ratio
1	92.8	123.2	0.75
2	96.0	115.5	0.83
3	168.0	150.4	1.12
4	86.4	88.0	0.98
5	121.6	107.0	1.14
6	86.4	78.0	1.08
7	179.2	129.6	1.38
8	137.6	110.4	1.25
9	118.4	105.6	1.12
10	115.2	118.4	0.97
11	121.6	99.2	1.23
Mean $\pm$ S.D.	120.29 $\pm$ 31.18	111.39 $\pm$ 19.81	1.08 $\pm$ .19

ng/mg pituitary tissue, respectively) as indicated by the ratio of RRA/RIA of 1.08 $\pm$ 0.056 indicating that similar quantities of TSH activity were being detected by both assays.

Column Chromatography of a Bovine Pituitary Extract

In order to further characterize the RRA for bTSH, 0.5 ml of bovine pituitary extract number 4 containing 4.32 and 4.4 μg bTSH activity as detected by RRA and RIA respectively, was brought to a volume of 1 ml in Tris-HCl buffer, pH 7.2, and chromatographed on a 1 x 90 cm column of Sephadex G-100. The 2.2 ml eluant fractions were appropriately diluted in 0.025 M Tris-HCl buffer containing 10mM $MgCl_2$, and assayed by both RRA and RIA for bTSH activity.

Figure 6 shows the protein elution of the bovine pituitary extract pattern and the peaks of bTSH in the eluant fractions as detected by both RRA and RIA. The TSH activity, as detected by both assay systems, was in fractions 17-28 indicating that activities of the same molecular size were being monitored by both assays. The RRA for bTSH detected 3.45 μg bTSH activity in the eluant fractions (80.0% recovery) and the RIA for bTSH detected 3.18 μg bTSH activity (72.4% recovery). The similarity in the percentage of recoveries for bTSH activity by both RRA and RIA further indicated that one bTSH activity was being monitored by both assay systems.

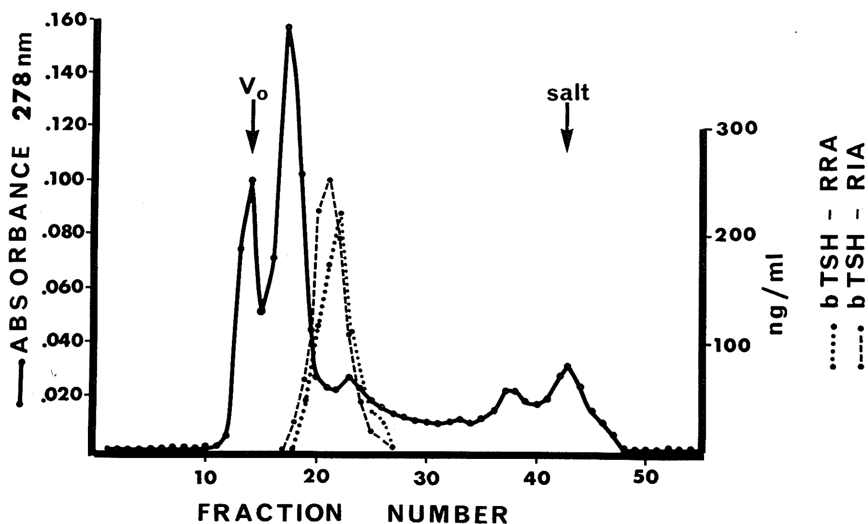

Figure 6 : Elution pattern of a bovine pituitary gland extract after gel filtration on a column of Sephadex G-100 (1x90 cm). Protein was determined by absorbance at 278 nm (●—●). The void volume and salt peak of the column were determined by chromatographing Blue Dextran and Na^{125}-I on the same column, and TSH activity in the eluant fractions (50 drops = 2.2 ml/tube) was determined by both RRA (••••••••••) and RIA (-----).

Some Applications of the RRA for TSH

TSH Activity from Various Species

Since bTSH and rTSH of similar biological potencies displaced on an equal weight basis in the RRA for bTSH, pituitary extracts were prepared from dog, rabbit, rat, guinea pig, turkey and chicken in order to see if the RRA for bTSH could be utilized to monitor TSH activity in different species. The pituitary extracts were appropriately diluted in 0.025 M Tris-HCl buffer, pH 7.2 containing 10mM $MgCl_2$, and assayed by both the RRA and RIA for bTSH.

As is shown in Table 4, TSH activity was detected by RRA in every pituitary extract assayed; however, by RIA, TSH activity was only detected in the dog pituitary extract, possibly due to a small amount of cross- reactivity in the RIA resulting from the large quantity of TSH activity present (700 ng/mg pituitary tissue as detected by RRA).

Gel Filtration of Pituitary Extracts from Various Species

In order to determine if there was any similarity in molecular size between the TSH activities in cow pituitary extracts and the pituitary extracts of other species, 1 ml each of extract from dog, rabbit, rat, guinea pig, turkey and chicken were chromatographed individually onto a 1x90 cm column of Sephadex G-100.

The protein elution pattern, as monitored by the absorbance of the eluant fractions at 278 nm, was similar in all cases to the protein elution pattern of the cow

TABLE 4 : Results of assay of pituitary extracts from various species by the radioreceptor and radioimmunoassays for bTSH.

Pituitary Extract	TSH Activity ng/mg tissue	
	RRA	RIA
Cattle	120.3	111.4
Dog	700	19
Rabbit	64	N.D.
Rat	90	N.D.
Guinea Pig	80	N.D.
Turkey	70	N.D.
Chicken	24	N.D.*

*N.D. denotes non detectable.

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pituitary extract shown in figure 6.

The eluant fractions were then appropriately diluted in 0.025M Tris-HCl buffer, pH 7.2 containing 10mM $MgCl_2$, and assayed by the RRA for bTSH. In each species, TSH activity (as detected by RRA) was found in fractions 17-28, indicating an inter-species similarity in the molecular size of TSH.

Eluant fractions from each species were then pooled, lyophillized and stored at 4° until required for further studies.

Interaction of TSH Activity from Various Species in the RRA for bTSH.

The lyophillized fractions of the TSH activity from the various species were further characterized by dissolving them at a concentration of 100,000 ng dry weight/ ml of 0.1M NH_4HCO_3, and then diluting appropriately in 0.025M Tris-HCl buffer for the RRA for bTSH.

As can be seen in figure 7, the lyophillized pituitary fractions containing TSH activity from cow, dog, rabbit, rat, guinea pig, turkey and chicken, after gel filtration on Sephadex G-100, interacted in a parallel manner to the bTSH standard in this RRA system, indicating that TSH from different species interacted in a similar manner with the porcine thyroid membrane receptor; however, on a weight basis, this activity was only 1/1000 the potency of the purified bTSH.

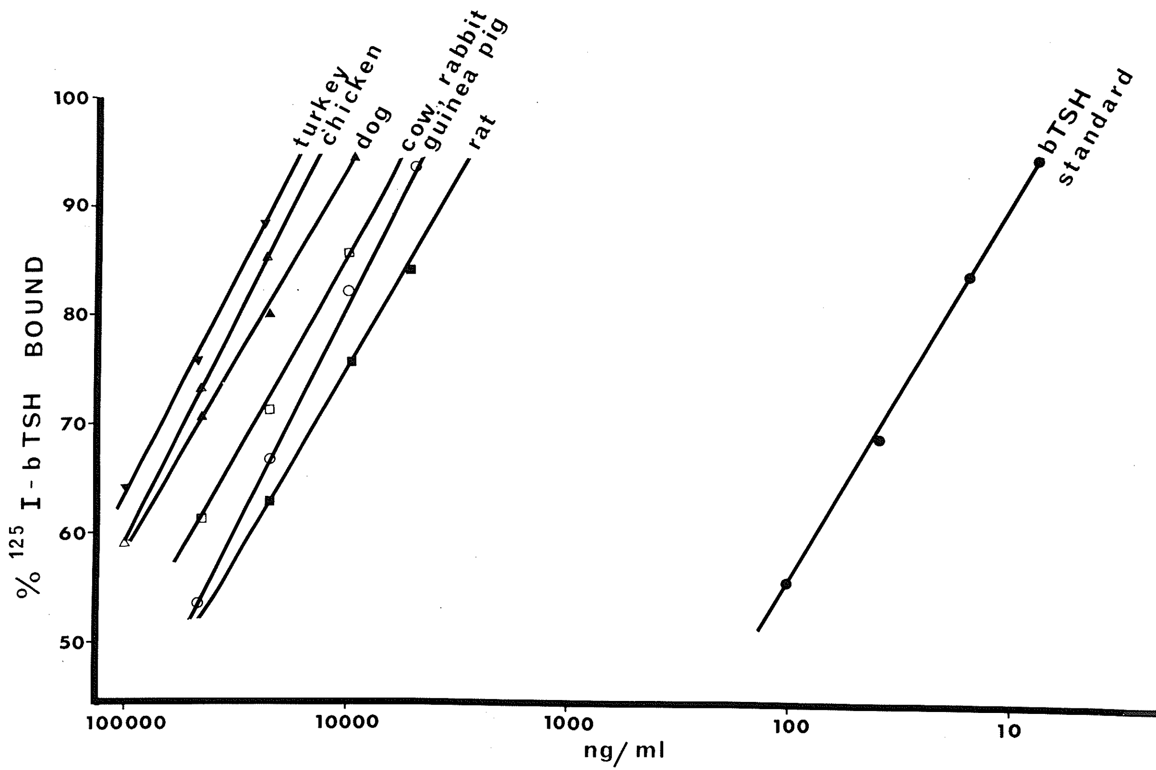

Figure 7 : Dose-response curves of radioreceptor assays for purified bTSH standard and the TSH activities in pituitary extracts from various species after gel filtration on Sephadex G-100.

Polyacrylamide Gel Electrophoresis of TSH Activities from Various Species.

Since there appeared to be an interspecies similarity in molecular size and interaction in the RRA, the TSH activities from cow, dog, rabbit, rat, guinea pig and turkey pituitary extracts were further characterized by polyacrylamide gel electrophoresis. Lyophillized fractions containing TSH activity were weighed out (150-200μg) and dissolved in 50μl of 0.012M sodium glycinate buffer, pH 9.5, and subjected to polyacrylamide gel electrophoresis. The electrophoretic mobilities of the TSH activity from different species were then compared to that of bTSH prepared in an identical manner.

The 6 cm gels were carefully cut into twelve 5mm segments, the protein in each gel segment was eluted with 0.5% NH_4HCO_3 buffer and diluted appropriately in 0.025M Tris-HCl buffer, pH 7.2 containing 0.1% BSA, and assayed for TSH activity with the RRA for bTSH.

As can be seen in figure 8, the major peak of bTSH activity was detected in gel segment 6; where as the TSH activity from the cow pituitary was mainly in gel segment 5. Variations in electrophoretic mobilities of TSH from different species are shown in figure 9. Dog and rat TSH activity was observed mainly in gel segment 6; guinea pig TSH activity was found in gel segment 3; rabbit TSH activity was dominent in gel segment 5; where as turkey TSH

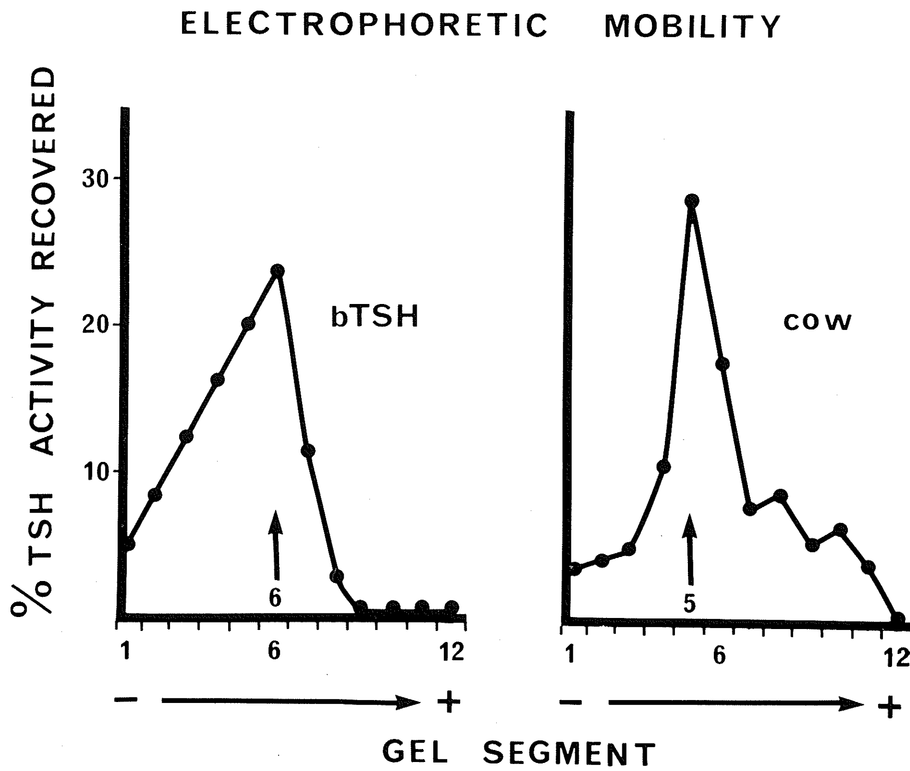

Figure 8 : Electrophoretic mobilibties of purified bTSH and TSH activity in crude pituitary extracts, after gel filtration on Sephadex G-100, by the RRA for bTSH.

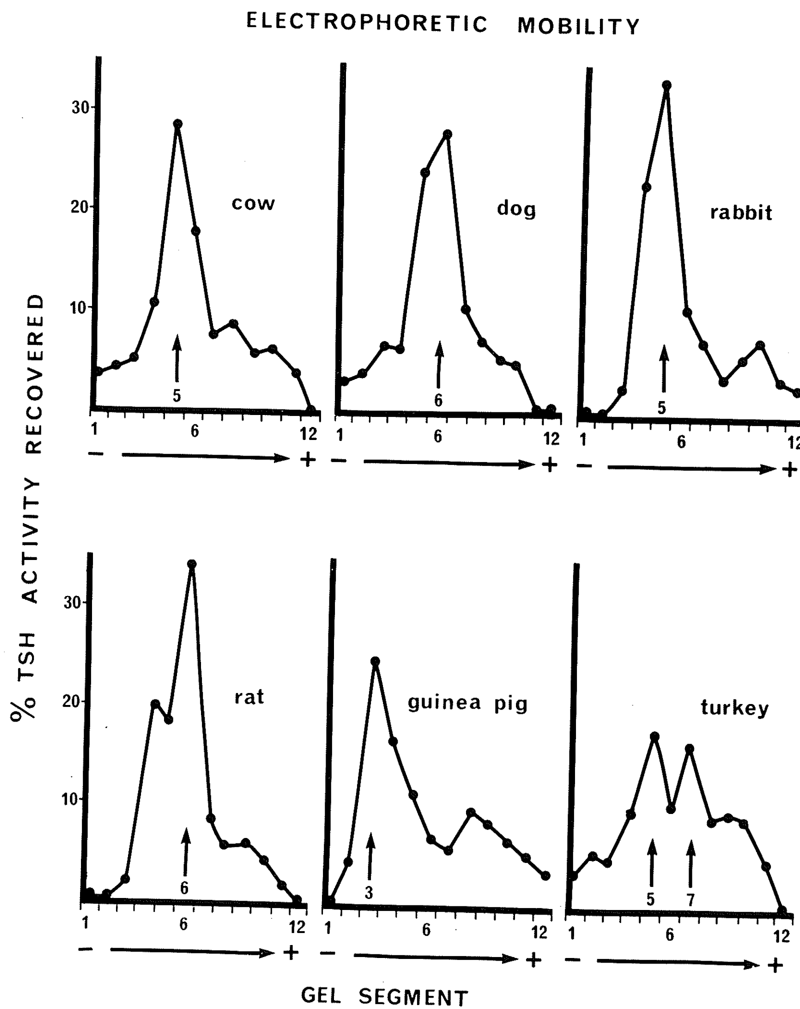

Figure 9 : Electrophoretic mobilities of TSH activity by RRA in crude pituitary extracts of various species after gel filtration on Sephadex G-100.

activity was equally divided between segments 5 and 7.

These observations indicated that although there was an interspecies similarity in the molecular size for TSH, the electrophoretic mobilities of TSH from different species varied slightly.

Characterization of Radioimmunoassays

Since it was necessary to know the levels at which the intact hormones cross-reacted in the homologous RIAs used in this study, the RIAs for bFSH, bFSHα, bFSHβ, bTSH, bTSHβ and bLHβ were characterized for their sensitivity and specificity.

Concentration of Polyethylene Glycol Required to Precipitate the Antibody-Hormone Complex

To determine the concentration needed to maximally precipitate the antibody-hormone complex with minimal precipitation of free ^{125}I-labelled hormone, polyethylene glycol was dissolved in PBS, pH 7.4, containing 0.1% BSA, at concentrations ranging from 5-40% (^{w}/v).

Reaction tubes containing 100 μl of PBS,pH 7.4, containing 0.1% BSA and 100 μl of ^{125}I-bFSH (25,000 CPM) in the presence and absence of 100 μl of RAS-bFSH-I-B2 (1:1000 dilution in the same buffer, were incubated overnight at room temperature. At the end of the incubation period, the reactions were stopped by adding 200 μl of RIA buffer, 500 μl of bovine-γ-globulins (dissolved at 2% ^{w}/v in RIA buffer) and 1 ml of polyethylene glycol at the various concentrations

to each tube. The tubes were immediately vortexed and centrifuged for 30 minutes (1,500x g at 4°) in an IEC-PR6000 centrifuge to separate the anti-body hormone complex. The supernatant was aspirated and the radioactivity in the pellet was counted in an automatic gamma counter.

Figure 10 shows that 22% polyethylene glycol maximally precipitated the antibody-hormone complex with minimal precipitation of free ^{125}I-bFSH. Experiments carried out using identical procedures as described above, showed that 22% polyethylene glycol was good for the separation of antibody-bound and free hormone for the radioimmunoassays for bFSHα, bFSHβ, bTSH, bTSHβ and bLHβ.

Titration of Antisera

For assay purposes, the various antisera were used at a titer which bound 35% of the total ^{125}I-labelled hormone in the incubation mixture in order to obtain the maximal sensitivity for the assay.

The titration curve for RAS-bFSH-I-B2 is shown in figure 11. The antiserum was diluted to the various dilutions in PBS, pH 7.4, containing 0.1% BSA. One hundred microliters of the antiserum of the different dilutions were incubated in duplicate with 100 μl of ^{125}I-bFSH (25,000 CPM in PBS, pH 7.4, containing 0.1% BSA) in the presence of 100μl of bFSH (at concentrations of 1 and 10 ng/ml) diluted in the same buffer. The RAS-bFSHI-B2 bound 35% of the ^{125}I-bFSH at a titer of 1:60,000.

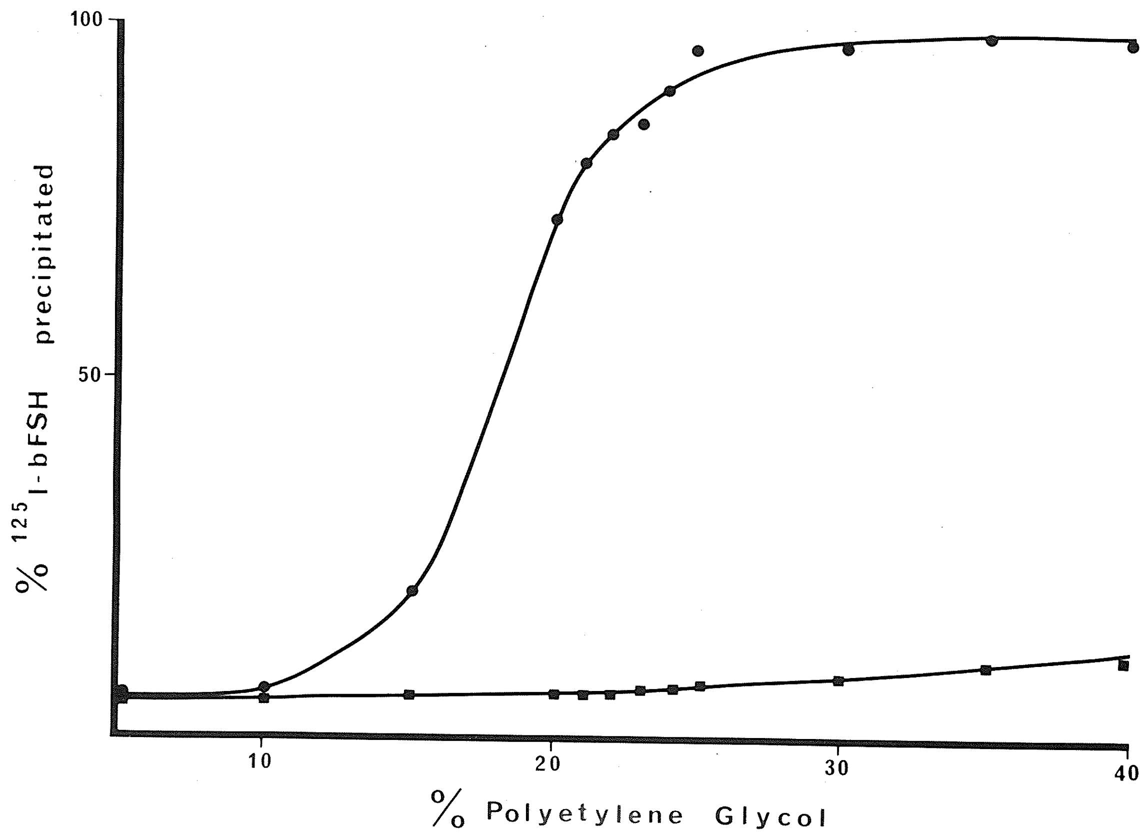

Figure 10 : Effects of different concentrations of polyehtylene glycol on the precipitation of antibody-bound ^{125}I-bFSH and free ^{125}I-bFSH tracer. ■—■ = free ^{125}I-bFSH tracer; ●—● = antibody-bound ^{125}I-bFSH.

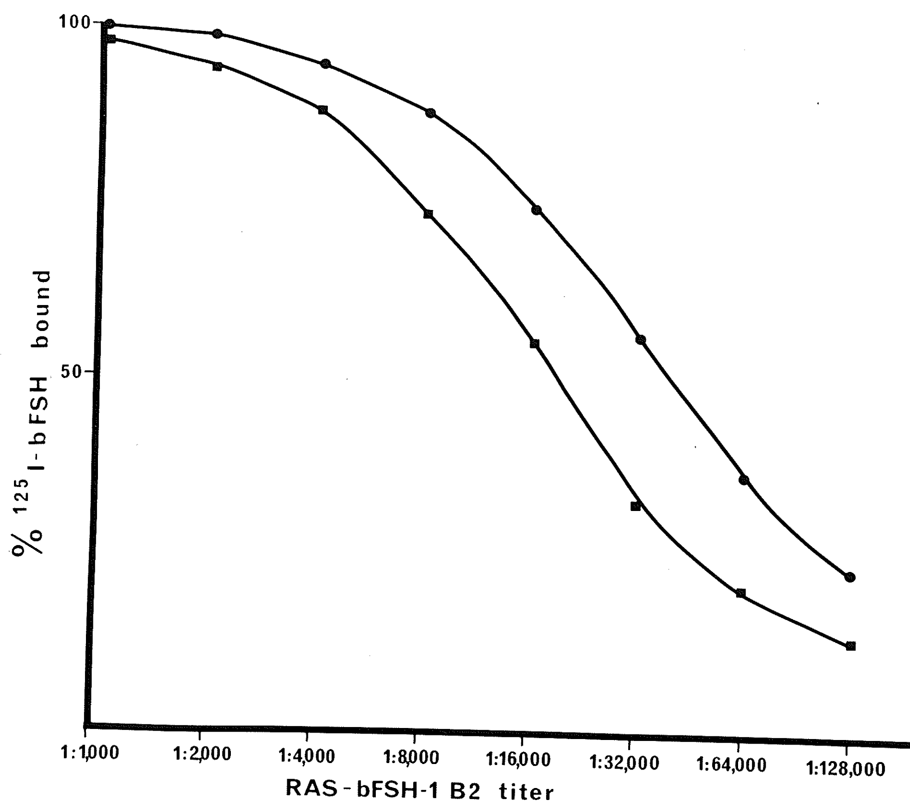

Figure 11: Titration of RAS-bFSHI-B2. Closed circles (●—●) are values for the binding of ^{125}I-bFSH in tubes containing 100μl of bFSH (ng/ml) at the various antiserum titers and closed squares (■—■) represent tubes containing 100 μl of bFSH (10 ng/ml) at the various antiserum titers.

Titration of all of the other antisera used in these studies was performed in an identical manner. The ^{125}I-labelled hormones used in the titrations and the titer of the specific antisers binding 35% of the tracer are summarized in table 5.

The Sensitivity and Specificity of the Homologous Radioimmunoassay for bFSH

Figure 12 shows that bFSH, at a concentration of lng/ml, significantly displaced the ^{125}I-bFSH. The cross-reactivity of all other hormones tested in the assay was $<$ 1%.

The Sensitivity and Specificity of the Homologous Radioimmunoassay for bFSHα.

Figure 13 shows that bFSHα, bLHα andbTSHα all displaced ^{125}I-bFSHα from RAS-bFSHα on an equal weight basis. The detectable range of the assay was from lng/ml to 50ng/ml for each α-subunit preparation. Intact bFSH,bLH and bTSH cross-reacted in a parallel manner to the α-subunit at levels of 10%. The cross reactivities of all other hormones tested was $<$ 1%.

The Sensitivity and Specificity of the Homologous Radioimmunoassay for bFSHβ

Figure 14 shows that bFSHβ at a concentration of 0.5 ng/ml displaced ^{125}I-bFSHβ from RAS-bFSHβ. Maximum displacement occurred and a concentration of 50 ng/ml of bFSHβ.Bovine FSH cross-reacted in a parallel manner to bFSHβ at a level of 5%. The cross reactivities of all other hormones tested was $<$ 1%.

TABLE 5: Determination of the titer of various antisera to obtain 35% binding. In cases where there is more than on antiserum for a given antigen, the first digit represents the rabbit number and the second digit indicates the bleeding number.

Rabbit Antiserum	Tracer Used	Titer of antiserum (bound 35% tracer)
bFSH I-1	^{125}I-bFSH	1:30,000
bFSH I-2	^{125}I-bFSH	1:60,000
bFSH I-3	^{125}I-bFSH	1:60,000
bFSH I-4	^{125}I-bFSH	1:70,000
bFSH I-5	^{125}I-bFSH	1:80,000
bFSHαI-1	^{125}I-bFSHα	1: 7,000
bFSHαI-2	^{125}I-bFSHα	1: 6,000
bFSHαI-3	^{125}I-bFSHα	1: 5,000
bFSHαI-4	^{125}I-bFSHα	1: 4,000
bFSHαII-1	^{125}I-bFSHα	-
bFSHαII-2	^{125}I-bFSHα	1: 6,000
bFSHαII-3	^{125}I-bFSHα	1: 7,000
bFSHαII-4	^{125}I-bFSHα	1: 5,000
bFSHβI-1	^{125}I-bFSHβ	1: 9,000
bFSHβI-2	^{125}I-bFSHβ	1: 5,000
bFSHβI-3	^{125}I-bFSHβ	-
bFSHβI-4	^{125}I-bFSHβ	-
bFSHβI-5	^{125}I-bFSHβ	1: 5,000
bFSHβI-6	^{125}I-bFSHβ	-
bFSHβI-7	^{125}I-bFSHβ	1: 5,000
bFSHβI-8	^{125}I-bFSHβ	1: 5,000
bFSHβII-1	^{125}I-bFSHβ	1: 5,000
bFSHβII-2	^{125}I-bFSHβ	1: 4,000
bFSHβII-3	^{125}I-bFSHβ	-
bFSHβII-4	^{125}I-bFSHβ	-
bTSH	^{125}I-bTSH	1:30,000
bTSHβ	^{125}I-bTSHβ	1:30,000
bLHβ	^{125}I-bLHβ	1:20,000

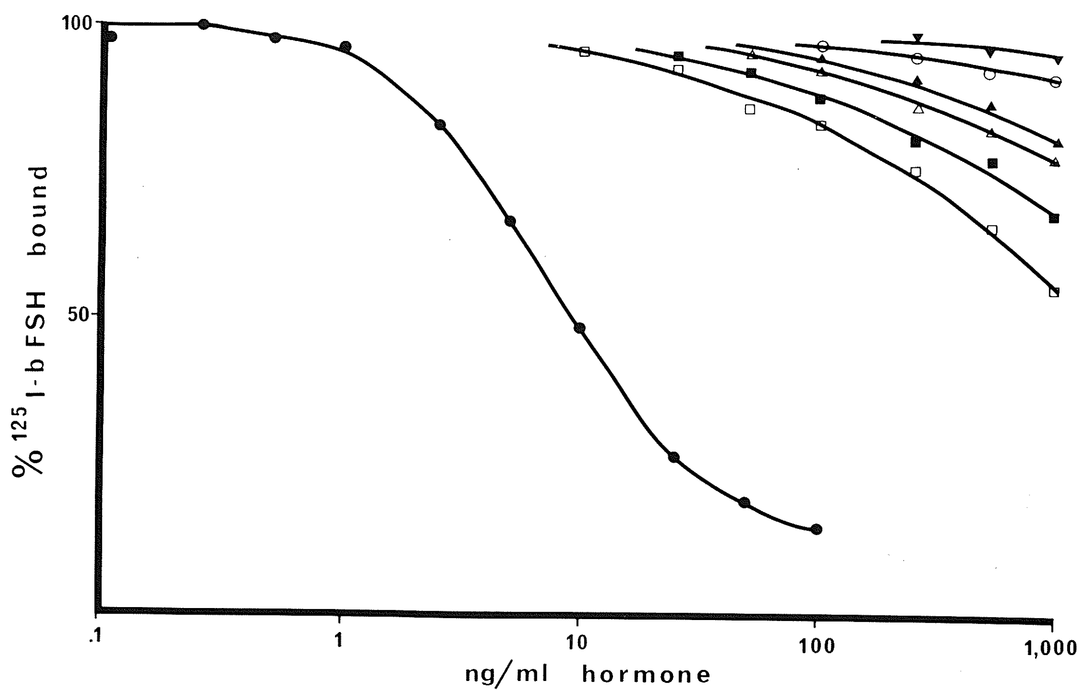

Figure 12 : Standard curves for bFSH and other protein and polypeptide hormones in the RIA using RAS-bFSH showing the sensitivity and specificity of the RIA system. ●—● = bFSH; ▼—▼ = bTSHβ, bLHβ, oPRL, bGH, pInsulin; ○—○ = bLHα; ▲—▲ = bTSHα; △—△ = bFSHβ; ■—■ = bFSHα, bLH; □—□ = bTSH.

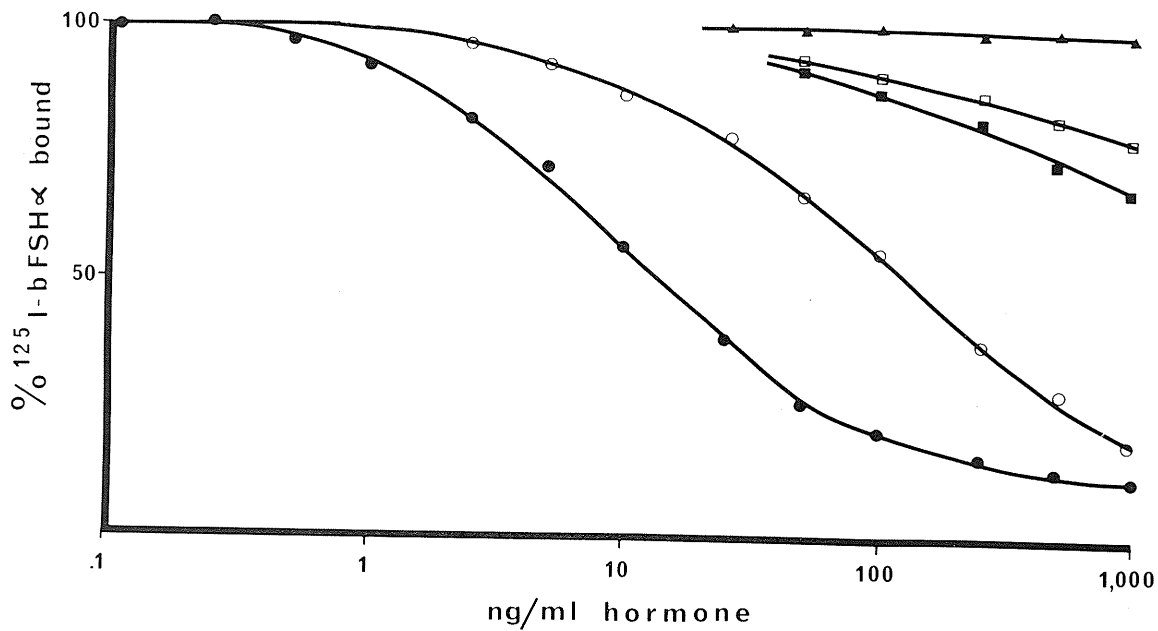

Figure 13: Standard curves for bFSHα and other protein and polypeptide hormones in the RIA using RAS-bFSHα showing the sensitivity and specificity of the RIA system. ●—● = bFSHα, bLHα, bTSHα; ○—○ = bFSH, bLH, bTSH; □—□ = bFSHβ, bTSHβ; ■—■ = bLHβ; ▲—▲ = bGH, oPRL, pInsulin.

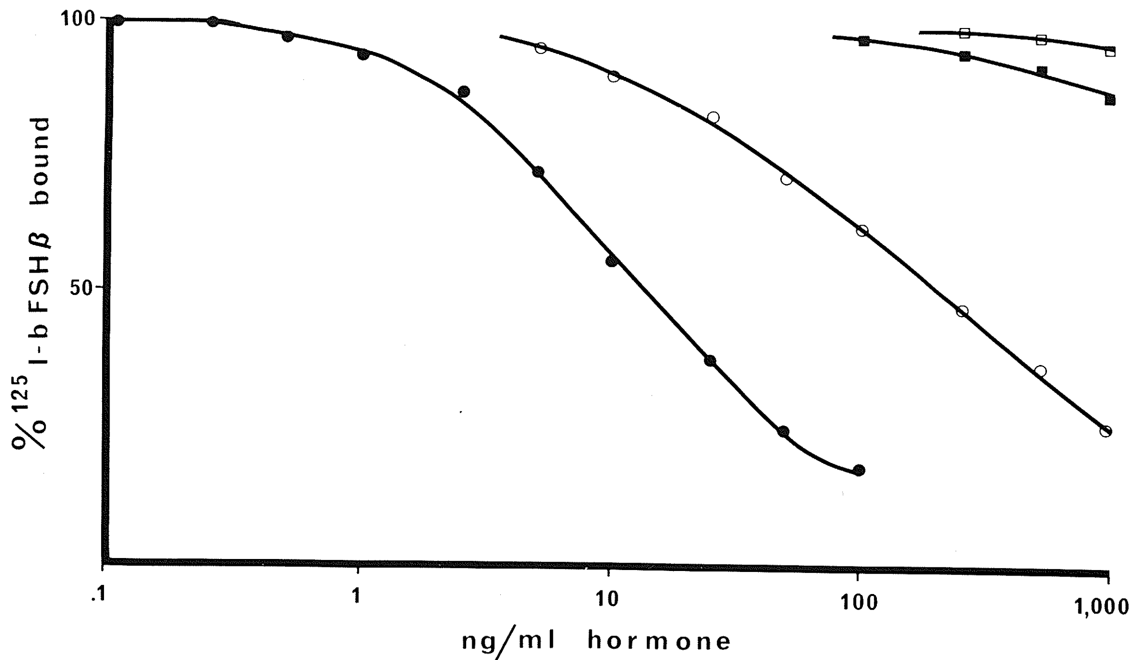

Figure 14 : Standard curves for bFSHβ and other protein and polypeptide hormones in the RIA using RAS-bFSHβ showing the sensitivity and specificity of the RIA system. ●——● = bFSHβ; ○——○ = bFSH; ■——■ = bFSHα, bTSH; □——□ = bLH, bLHα, bLHβ, bTSHα, bTSHβ, oPRL, bGH, pInsulin.

The Sensitivity and Specifcity of the Homologous Radioimmunoassay for bTSH

Bovine TSH, at a concentration of lng/ml, displaced ^{125}I-bTSH from RAS-bTSH (Figure 15). Maximum displacement occurred at a concentration of 100 ng/ml. In the assay system, bTSHβ cross-reacted in a parallel manner at a level of 10%. The cross-reactivities of all other hormones tested was < 1%.

The Sensitivity and Specificity of the Homologous Radioimmunoassay for bTSHβ

Bovine TSHβ, at a concentration of 0.5 ng/ml, displaced ^{125}I-bTSHβ from RAS-bTSHβ (figure 16). Maximum displacement occured at a concentration of 25 ng/ml. Bovine TSH cross-reacted at a level of 25% in a parallel manner to bTSHβ. The cross-reactivities of all other hormones tested was < 1%.

The Sensitivity and Specificity of the Homologous Radioimmunoassay for bLHβ.

Bovine LHβ at a concentration of 0.5 ng/ml, displaced ^{125}I-bLHβ from RAS-bLHβ (figure 17). Maximum displacement occured at a bLHβ concentrationof 50 ng/ml. Bovine LH cross-reacted at a level of 15% in a parallel manner to bLHβ. The cross-reactivites of all other hormones tested was < 1%.

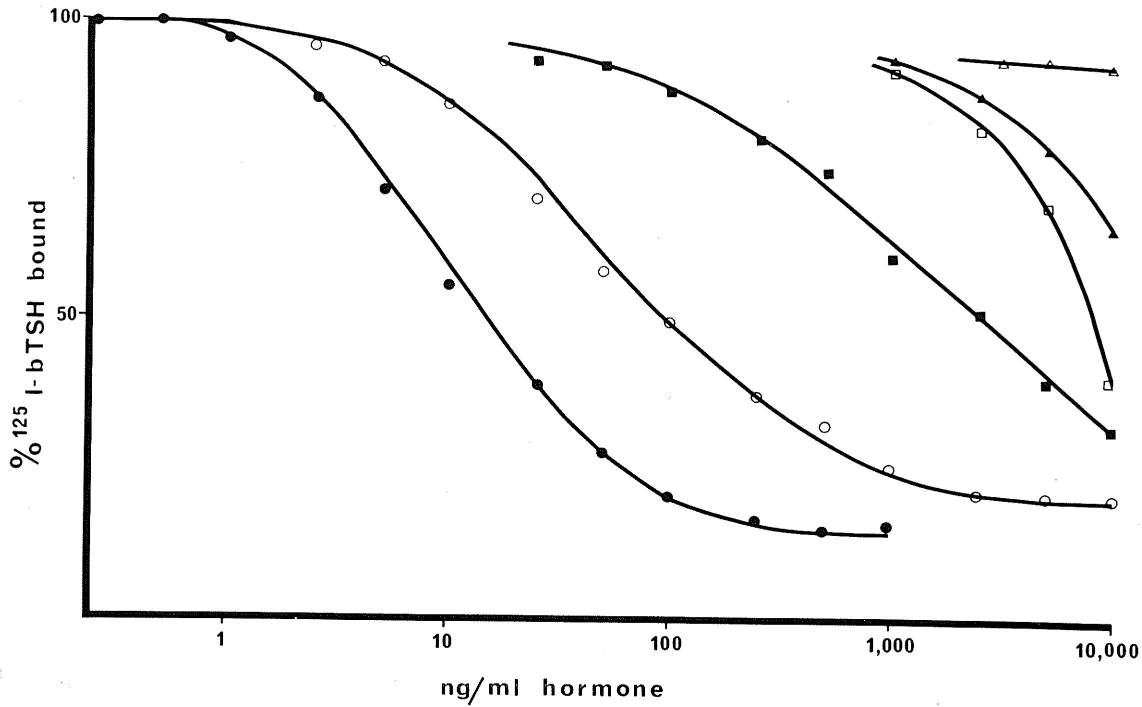

Figure 15 : Standard curves for bTSH and other protein and polypeptide hormones in the RIA using RAS-bTSH showing the sensitivity and specificity of the RIA system. ●—● = bTSH; ○—○ = bTSHβ; ■—■ = bTSHα; □—□ = bLH; ▲—▲ = bFSH, bGH; △—△ = bLHα, bLHβ, oPRL, pInsulin.

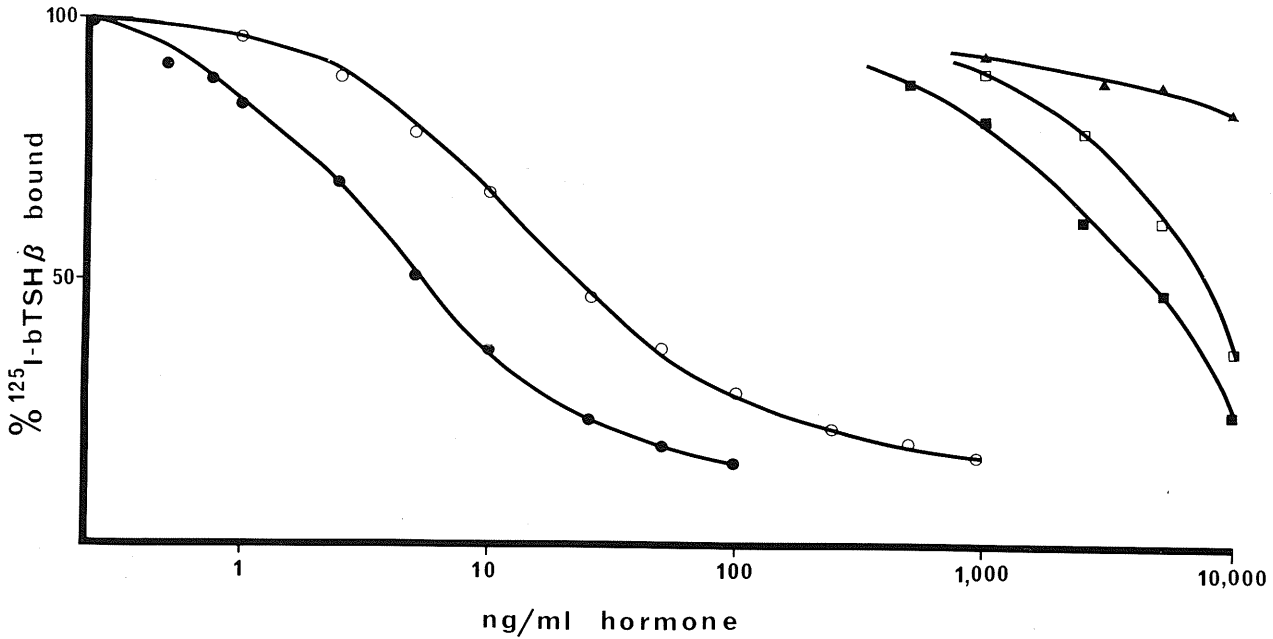

Figure 16 ; Standard curves for bTSHβ and other protein and polypeptide hormones in the RIA using RAS-bTSHβ showing the sensitivity and specificity of the RIA system. ●—● = bTSHβ; ○—○ = bTSH; ▲—▲ = bFSH, bLHα, bLHβ, oPRL, pInsulin; □—□ = bLH, bGH; ■—■ = bTSHα.

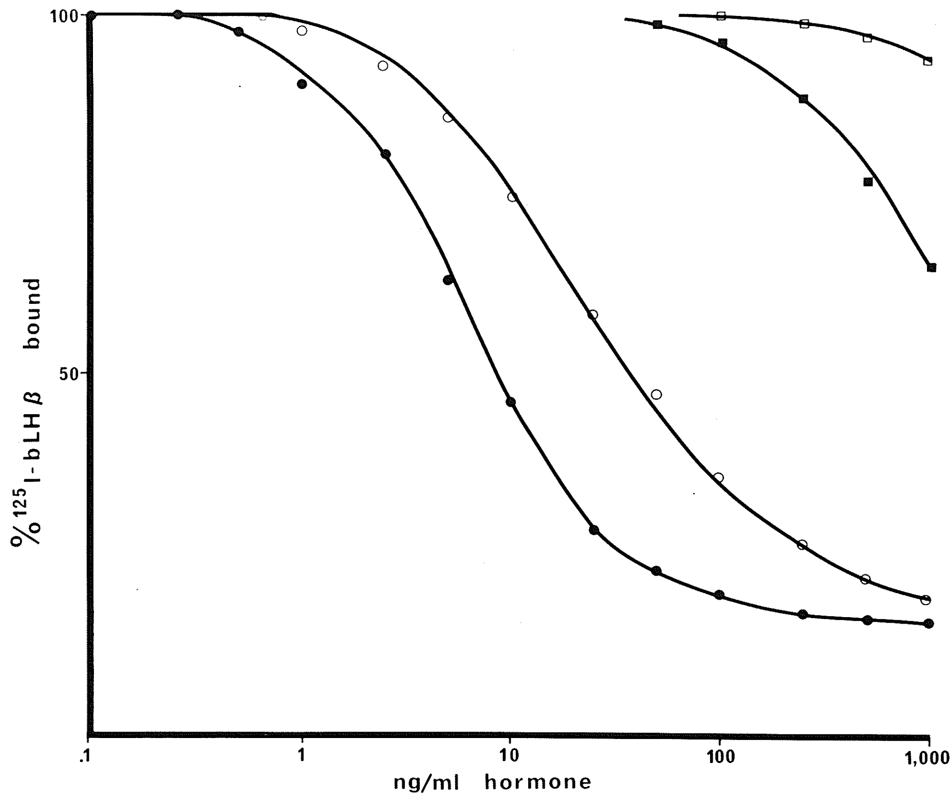

Figure 17 : Standard curves for bLHβ and other protein and polypeptide hormones in the RIA using RAS-bLHβ showing the sensitivity and specificity of the RIA system. ●—● = bLHβ, ○—○ = bLH; ■—■ = bFSH, bLHα, bGH; □—□ = bTSH, bTSHα, bTSHβ, bFSHβ, bFSH .

Validation of Values Obtained from Assay of Pituitary Extracts with the Various Assay Systems

In most of the published reports on studies of LH, FSH, TSH and subunit levels in fetal pituitary glands (21,35,37,41), the values for the hormones as determined by RIAs did not take into account the fact that the presence of anyone of these glycoprotein hormones in the extracts cross-reacted with one another in any RIA system for anyone of these hormones. In those studies, it was assumed that the cross-reactivites of others hormones in the RIAs was negligible after dilution of the pituitary extract. This assumption was not valid, as it has been shown (figures 12,13,14,15 ,16,17) that the presence of relatively small amounts of other glycoprotein hormones in a RIA cross-reacted significantly, and could result in a higher value for the hormone being assayed than was actually present. In this study, this problem became more significant in assaying a relatively small amount of α-subunit by RIA in the presence of a relatively large quantity of bLH in the pituitary extract.

In another study of TSH, TSHα and TSHβ levels in 5 pituitary glands (31) the hormones and the free subunit were separated by gel filtration of pituitary extracts on Sephadex G-100 and monitoring the eluant fractions with the various RIA systems. It is obvious that gel filtration of a relatively large number of samples is not a

practical solution to overcoming the problem of cross-reactivities in RIAs.

To overcome this problem of cross-reactivities in the RIA systems, the pituitary extracts were first assayed by specific RRA systems for bLH, bFSH and bTSH. In the RRAs, no cross-reaction was observed between any of these hormones. For the quantitation of α and β subunits, RIAs were used in which the cross-reactivities caused by different intact hormones would result in erroneous values. If the cross-reactivities caused by the intact hormones bLH, bFSH and bTSH had been precalculated in a particular RIA system, for example, the RIA for the α-subunit, valid values for the amount of α-subunit present could be obtained by simply subtracting the cross-reactivities due to the already determined amounts of bLH, bFSH and bTSH by the specific RRAs from the overestimated values of the subunit by the RIA.

In order to validate the system of caluclations outlined above to determine the quantities of hormones present in fetal pituitary glands, extracts from 4 different fetal pituitary glands (referred to as A,B,C, and D, respectively) were assayed by RRAs for bLH, bFSH and bTSH. The same samples were then assayed with the RIAs for the various subunits and the cross-reactivities due to the present of intact bLH, bFSH and bTSH were calculated and subtracted to obtain the absolute values for

the quantity of the subunits.

One milliliter of each pituitary extract was then gel filtered on a 2x110cm. column of Sephadex G-100 in order to separate the intact hormones and the subunits. Samples of standard purified bLH, bLHα, bFSHα, bTSHα and bFSH were each dissolved in 1 ml of 0.1M NH_4HCO_3, and individually chromatographed on the same Sephadex G-100 column. The eluant fractions (1.15 ml) were appropriately diluted in RRA buffer (0.025M Tris-HCl, pH 7.2, containing 0.1% BSA) or RIA buffer (PBS, pH 7.2, containing 0.1% BSA) and assayed by the appropriate RRA for intact hormones or RIA for the subunits to determine the relative elution profile and percentage of recovery for each hormone. After gel filtration on Sephadex G-100, elution volumes and percentages of recoveries of the purified hormone preparations and the hormonal activities in the pituitary extracts as determined previously by RRAs for the intact hormones and RIAs (subtracting cross-reactivities) for α and β subunits were very similar (Table 6). This indicated the validity of the values obtained for intact hormones (by assay of the crude pituitary extracts by the RRAs for bLH, bFSH and bTSH) and their α and β subunits (by subtracting the cross-reactivities resulting from the presence of intact hormones in the subunit RIAs) in the fetal pituitary extracts.

After gel filtration of 1 ml of bLH (53.4 μg) on Sephadex G-100, 41.3 μg bLH activity (77.4% recovery) as

TABLE 6 : Percentage of recoveries for various hormones after gel filtration on Sephadex G-100. The amount of purified hormone added to the column was determined by weighing the samples on a Cahn Model 4100 Electrobalance. Determination of quantities of intact hormone in fetal pituitary extracts was by the appropriate RRA. The α-subunit activity in fetal pituitary extracts was estimated as outlined in the CAlculation ofSubunit Activity section Methods and Materials. Quantity of hormone recovered was determined by assaying the eluant fractions. Intact glycoprotein hormone was detected by the appropriate RRA. α-subunit activity was detected by the RIA for bFSHα.

Sample	Quantity present (μg)	Quantity recovered (μg)	%Recovered
bLH	53.4	41.3	77.4
bLHα	67.4	45.5	67.5
bFSHα	43.2	27.3	63.1
bTSHα	70.0	40.4	57.7
bFSHβ	28.4	16.9	59.4
f.pit.ex.*A bLH	33.6	20.7	61.5
f.pit.ex. B bLH	7.7	5.0	65.4
f.pit.ex. D bFSH	15.8	9.3	58.8
f.pit.ex. D bTSH	11.6	5.4	45.3
f.pit.ex. A αsubunit	16.9	12.7	75.5
f.pit.ex. B αsubunit	10.0	5.1	51.0
f.pit.ex. C αsubunit	8.2	7.0	82.0

* f.pit.ex. denotes fetal pituitary extract.

measured by the RRA for bLH, was recovered in eluant fractions 101-134 (figure 18). After gel filtration of 1 ml of bLHα (67.4 μg) on the same column, 45.5 μg bLHα activity (67.5% recovery) as measured by the RIA for bFSHα, was recovered in eluant fractions 134-170 (Figure 18).

After gel filtration of 1 ml of fetal pituitary extract A, containing 33.6 μg bLH as determined by the RRA for bLH, and 16.88 μg free α-subunit, as determined by the RIA for bFSHα after cross-reactivities due to the presence of intact bLH, bFSH and bTSH had been subtracted, 20.7 μg bLH activity (61.5% recovery) as measured by the RRA for bLH was recovered in eluant fractions 105-134 and 12.7 μg free α-subunit (75.5% recovery) as measured by the RIA for bFSHα was recovered in eluant fractions 134-170 (figure 19).

After gel filtration of 1 ml of pituitary extract D, the percentage recovery of bTSH, bFSH and free α-subunit were also similar (figure 20).

Table 6 summarizes the quantities of hormones chromatographed and the percentage of recovery for each hormone.

Eluant fractions containing free α-subunit after gel filtration of bovine fetal pituitary extracts were pooled, lyophillized and stored at 4° for further studies.

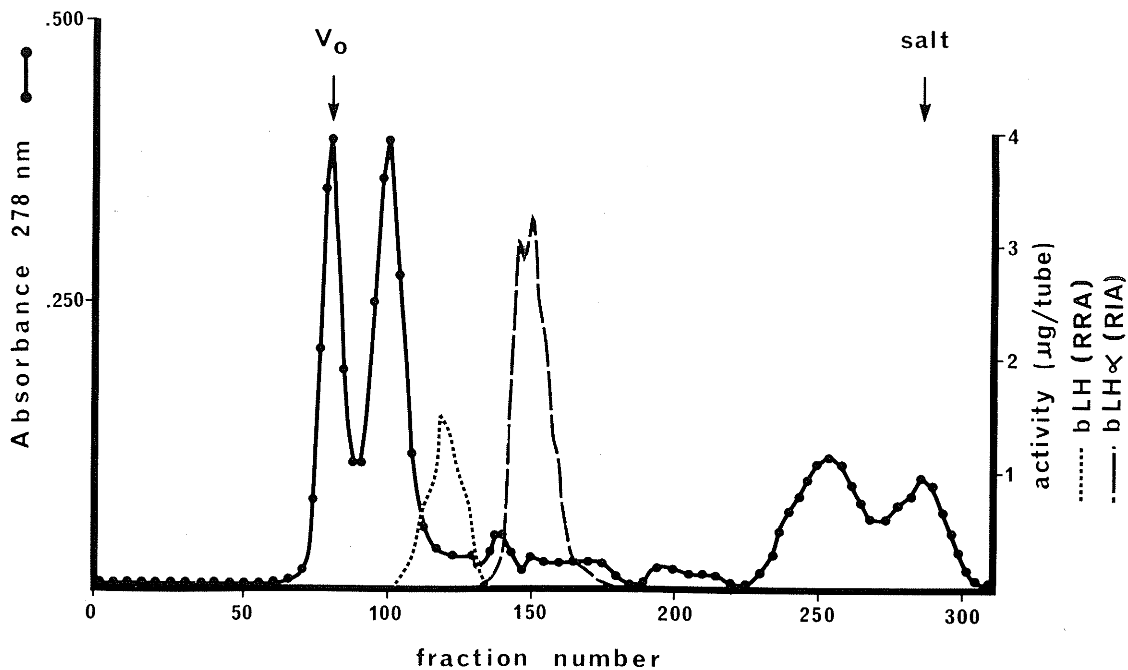

Figure 18 : Elution pattern of bovine fetal pituitary extract D after gel filtration on a column of Sephadex G-100 (2x10 cm). Protein was determined by absorbance at 278 nm (●—●). The void volume and salt peak were determined by chromatographing Blue Dextran and Na-^{125}I on the same column; LH activity in the eluant fractions (25 drops = 1.15 ml/tube) was determined by the RRA for bLH (-----); and LHα activity in the eluant fractions was determined by the RIA for α-subunit (— — —).

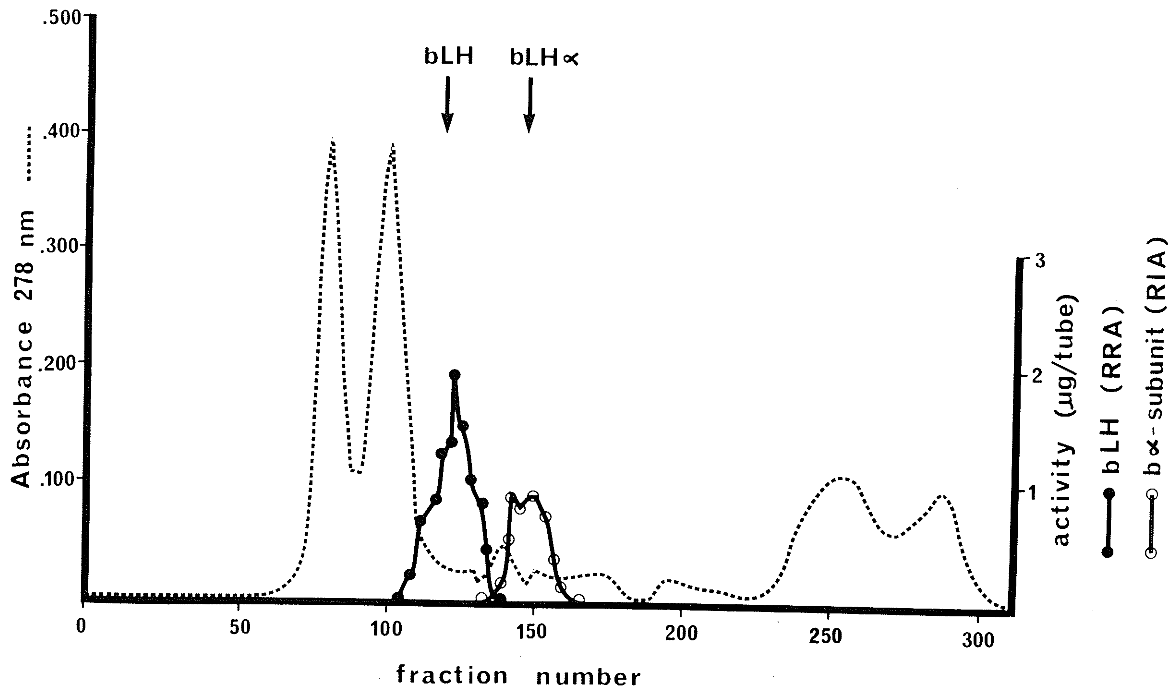

Figure 19 : Elution pattern of bovine fetal pituitary extract D after gel filtration on a column of Sephadex G-100 (2x110 cm). Protein was determined by absorbance at 278 nm (----). Bold arrows (↓) indicate the relative elution positions of bLH and bLHα standards chromatographed on the same column; LH activity in the eluant fractions (25 drops = 1.15 ml/tube) was determined by the RRA for bLH (●—●); free α-subunit in the eluant fractions was determined by the RIA for α-subunit.

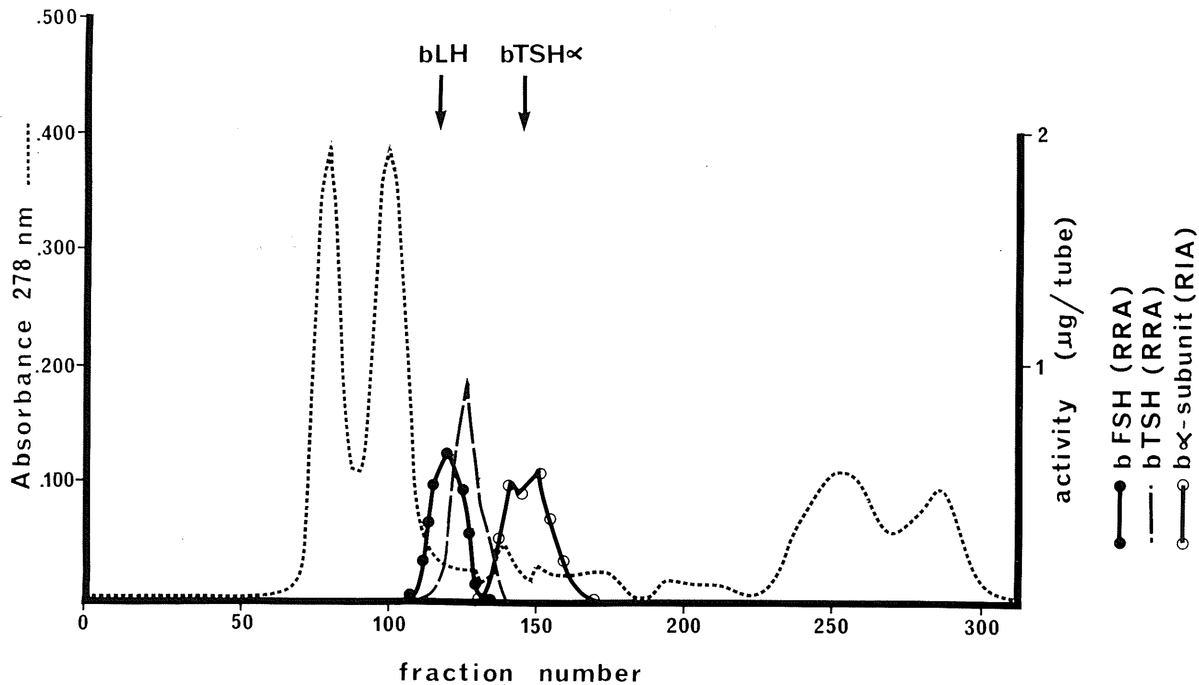

Figure 20 : Elution pattern of bovine fetal pituitary extract D after gel filtration on a column of Sephadex G-100 (2x110 cm). Protein was determined by absorbance at 278 nm (---). Bold arrows (↓) indicate the relative elution positions of bLH and bTSHα standards chromatographed on the same column; FSH activity in the eluant fractions (25 drops = 1.15 ml/tube) was determined by the RRA for bFSH; TSH activity in the eluant fractions was determined by the RRA for bFSH; free α-subunit in the eluant fractions was determined by the RIA for α-subunit.

Glycoprotein Hormones and Their α and β Subunits in Bovine Fetal Pituitary Glands

Relationship Between the Age of Fetuses and Fetal Pituitary Gland Weight

Figure 21 shows the relationship between the age of fetuses (months) and the wet weight of the fetal pituitary glands. By regression analysis of the data, it was observed that the relationship between the age of fetus and the weight of the fetal pituitary gland was linear ($p < 0.01$).

Bovine Fetal Pituitary Gland Content of Free α-Subunit

Figure 22 shows the relationship between the concentration of free α-subunit (ng/mg pituitary tissue) and fetal age (months) in male and female fetuses. The statistical analysis of this data is shown in Table 7. Pituitary glands from male fetuses < 3 3/4 months old (group 1), were found to have significantly more free α-subunit/mg pituitary tissue than the females of the corresponding age group (group 5) and males of 3 3/4 - 5 months old (group 2).

Figure 23 shows the relationship between the total amount of free α-subunit (μg/pituitary gland) and fetal age (months) in pituitary glands from male and female fetuses. Significant increases of free α-subunit between groups 2:3, 3:4 of the males, 6:7 and 7:8 of the females (Table 8), indicated that the content of free α-

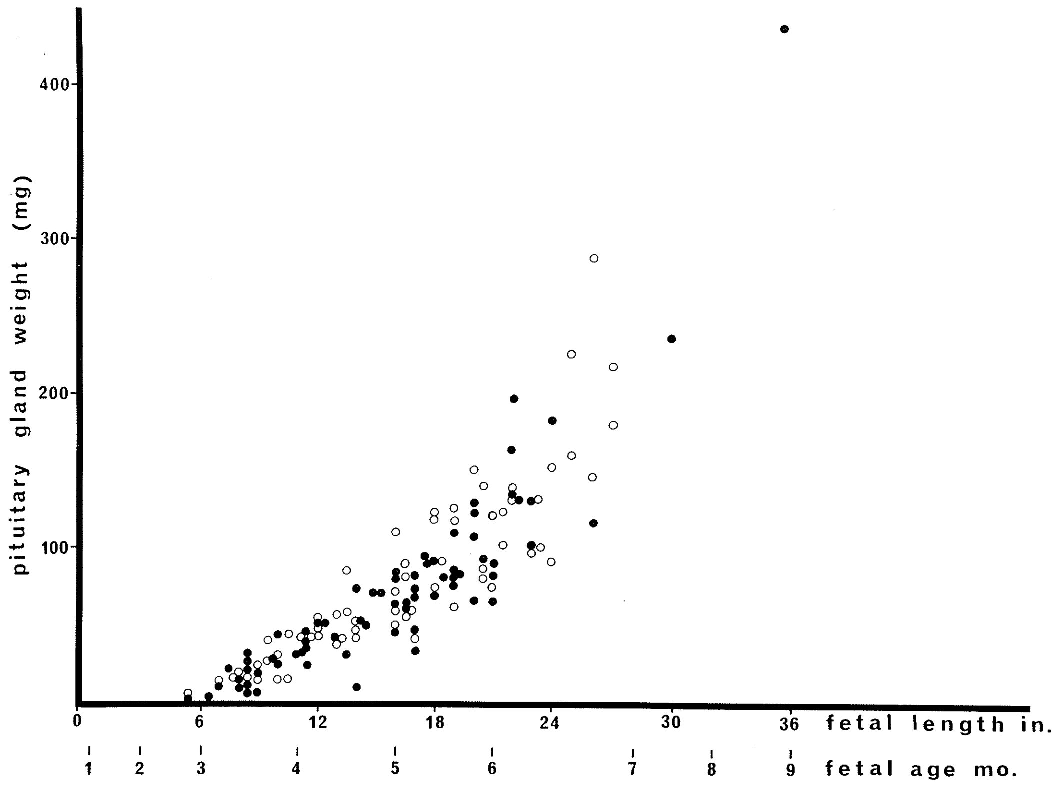

Figure 21: Relationship between age of fetuses (months) and weight of pituitary glands (mg). Closed circles (●) represent pituitary glands from male fetuses and open circles (○) represent pituitary glands from female fetuses.

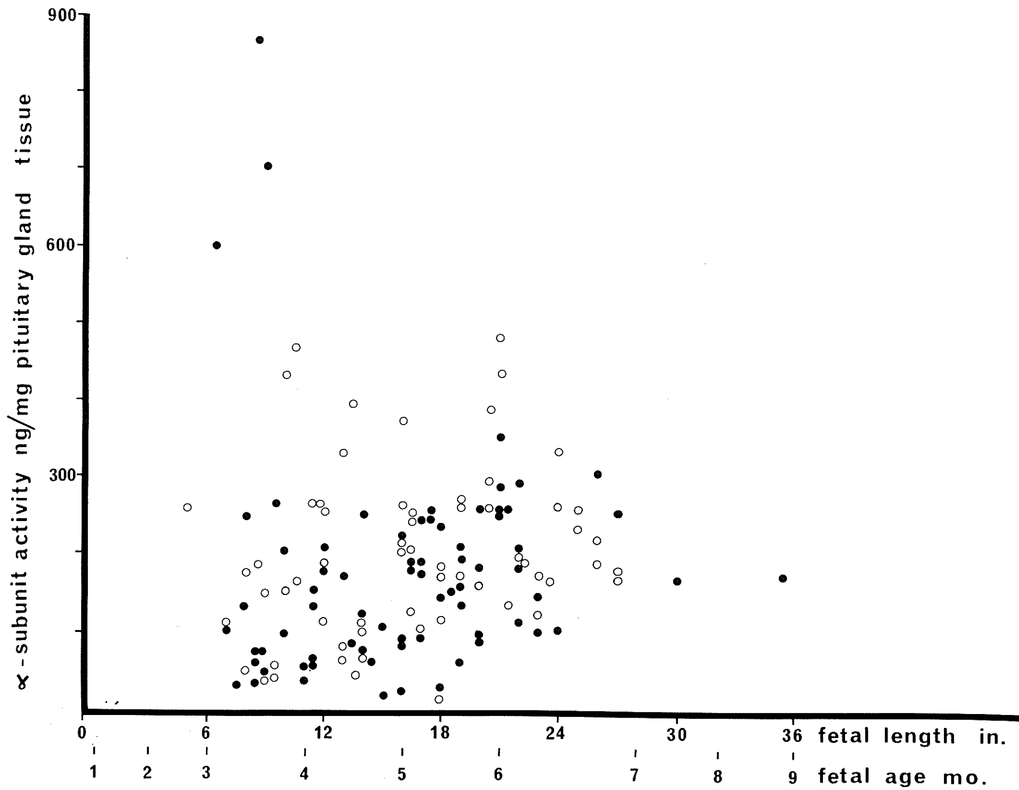

Figure 22 : Relationship between age of fetuses (months) and concentration of free α-subunit (ng/mg) in pituitary tissue as determined by assaying fetal pituitary extracts with the RIA for α-subunit and subtracting cross-reactivities due to the presence of intact bLH, bFSH and bTSH. Closed circles (●) represent values obtained for pituitary glands from male fetuses and open circles (○) represent values obtained for pituitary glands from female fetuses.

TABLE 7: Means and standard deviations of α-subunit activity, ng/mg pituitary tissue, in groups 1-8. Group 1 was found to have significantly higher values ($p < 0.05$) than both groups 2 and 3. No significant difference was found between other groups.

Sex	fetal age (months)*			
	< 3 3/4	3 3/4-5	5-6 1/4	> 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	247.4±284.7	114.3±67.2	177.3±82.8	177.6±73.5
	n = 13	n = 18	n = 30	n = 10
	Group 5	Group 6	Group 7	Group 8
females	116.6±80.5	201.9±137.5	231.4±111.1	205.4±54.4
	n = 9	n = 17	n = 23	n = 13

* < 3 3/4 months = < 9.9 inches fetal length
3 3/4 - 5 months = 10-15.9 inches fetal length
5 - 6 1/4 months = 10-21.9 inches fetal length
> 6 1/4 months = > 22 inches fetal length.

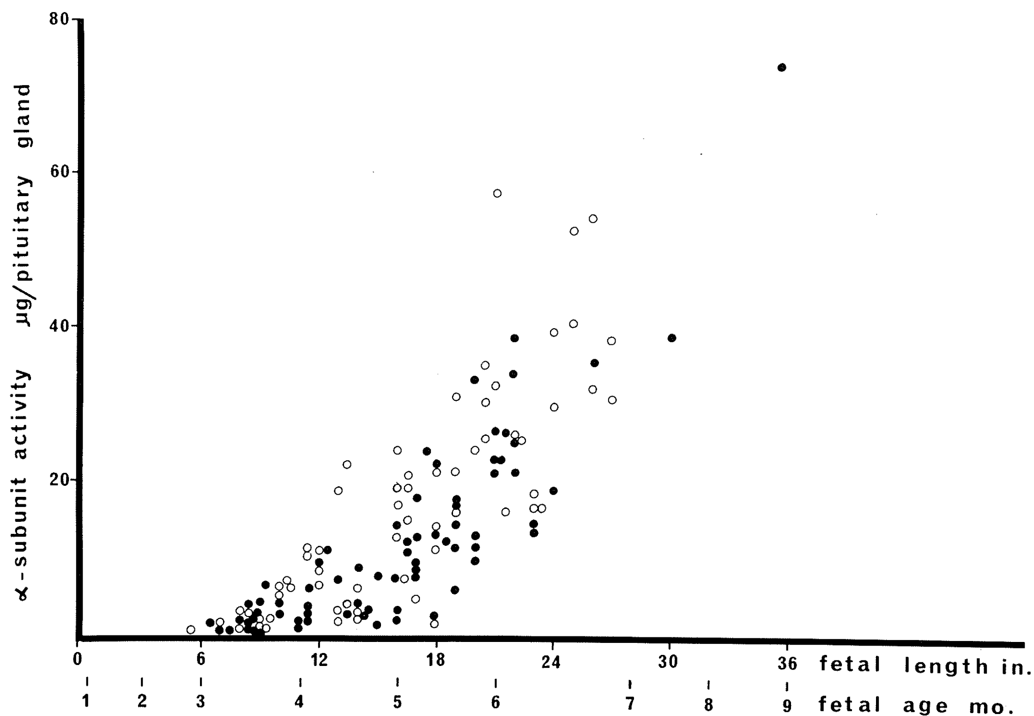

Figure 23 : Relationship between age of fetuses (months) and content of free α-subunit (μg) per pituitary glands as determined by assaying fetal pituitary extract s with the RIA for α-subunit and subtracting cross-reactivities due to the presence of intact bLH, bFSH and bTSH. Closed circles (●) represent values obtained for pituitary glands from male fetuses and open circles (O) represent values obtained for pituitary glands from female fetuses.

TABLE 8: Means and standard deviations of α-subunit activity, μg/pituitary, in groups 1-8. The amount of α-acitvity in fetal pituitaries increased with fetal age in both males and females as is indicated by significant increases between groups 2:3, 3:4, 6:7 and 7:8. Group 7 was found to have significantly more α -subunit activity than group 3.

	fetal age (months)*			
Sex	< 3 3/4	3 3/4 - 5	5 - 6 1/4	> 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	2.26±1.85	4.40±3.10	14.22±7.83	31.37±178.2
	n = 13	n = 18	n = 30	n = 10
	Group 5	Group 6	Group 7	Group 8
females	1.76±0.87	7.92±5.71	20.62±11.86	32.05±12.80
	n = 9	n = 17	n = 23	n = 13

*< 3 3/4 months = < 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months - 10. 21.9 inches fetal length.
> 6 1/4 months => 22 inches fetal length.

subunit in pituitary glands from both male and female fetuses increased with age. Pituitary glands from female fetuses, ages 5 - 6 1/4 months (group 7), were found to have a significantly higher free α-subunit content (20.62 ± 11.86 μg) than pituitary glands from male fetuses (14.22 ± 7.86 μg) of the corresponding age (group 3).

Bovine Fetal Pituitary Gland Content of LH

Figure 24 shows the relationship between the concentration of bLH (ng/mg pituitary tissue) and fetal age (months). Statistical analysis of the data (table 9), showed no difference between any groups, indicating that the amount of bLH/mg pituitary tissue remained relatively constant throughout gestation.

The amount of bLH (μg /pituitary gland) is shown in figure 25. Significant increases in pituitary bLH between groups 2:3, 3:4 of the males and 6:7, 7:8 of the females, indicated that the quantity of bLH/pituitary gland increased with fetal age (table 10). There was no difference in the pituitary gland content of bLH between males and females of corresponding age group.

Table 11 shows that there was no significant difference between any groups when the ratio of free α-subunit: bLH (per pituitary gland) was plotted against fetal age of both males and females.

Bovine Fetal Pituitary Gland Content of FSH

Figure 26 shows the relationship between the concentration of bFSH (ng/mg pituitary tissue) and fetal age

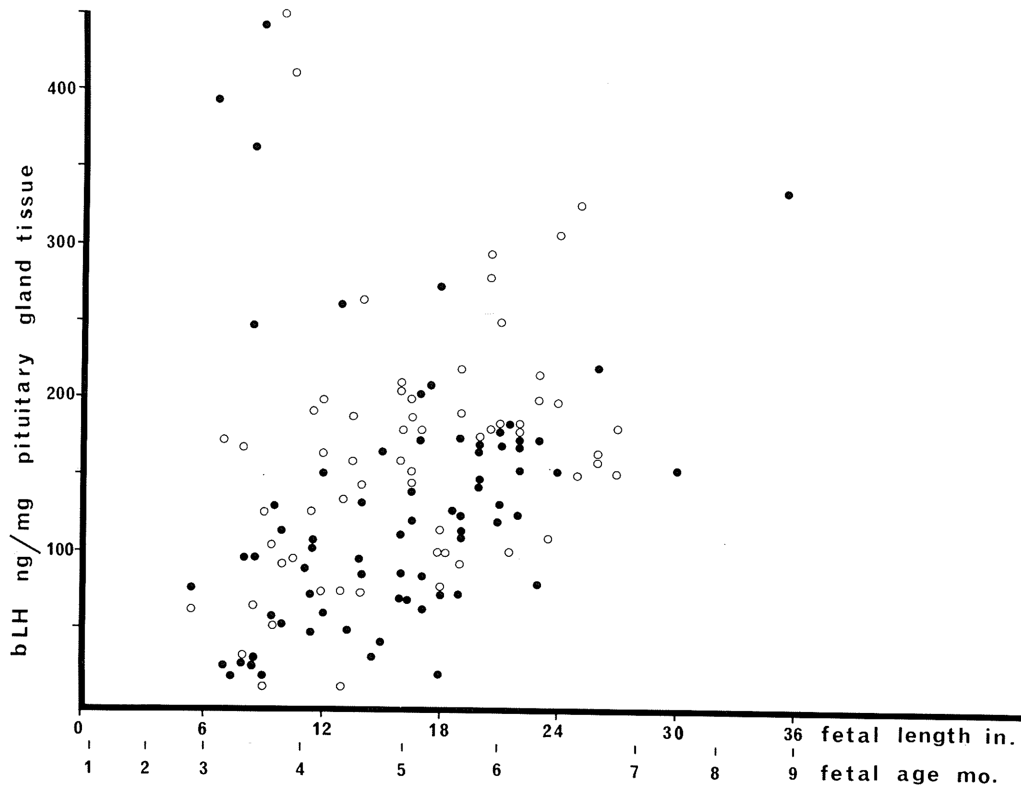

Figure 24 : Relationship between age of fetuses (months) and concentration of bLH (ng/mg) in pituitary tissue as determined by assaying fetal pituitary extracts with the RRA for bLH. Closed circles (●) represent bLH concentrations in pituitary glands from male fetuses and open circles (o) represent bLH concentrations in pituitary glands from female fetuses.

TABLE 9: Means and standard deviations of bLH, ng/mg pituitary tissue, in groups 1-8. No statistically significant differences were found between any groups.

	fetal age (months)*			
Sex	< 3/34	3 3/4 - 5	5 - 6 1/4	> 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	144.5±156.6 n = 14	90.0±57.0 n = 18	150.6±111.7 n = 30	173.5±67.7 n = 10
	Group 5	Group 6	Group 7	Group 8
females	89.0±57.3 n = 9	169.0±117.8 n = 17	173.4±59.0 n = 23	194.7±61.2 n = 13

* < 3 3/4 months = < 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
> 6 1/4 months = > 22 inches fetal length.

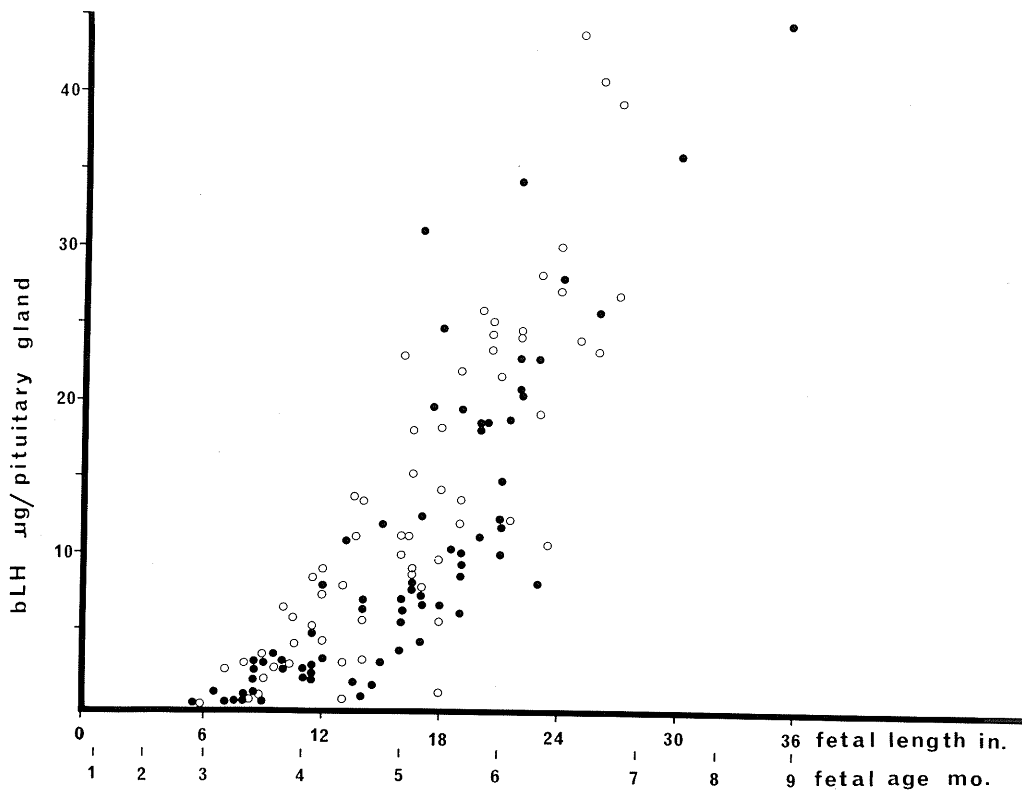

Figure 25 : Relationship between age of fetuses (months) and content of bLH (µg) per pituitary gland as determined by assaying fetal pituitary extracts with the RRA for bLH. Closed circles (●) represent bLH content in pituitary glands from male fetsuses and open circles (○) represent bLH content in pituitary glands from female fetuses.

Table 10 : Means and standard deviations of bLH, μg/pituitary gland. Statistically significant increases ($p < .05$) occur between groups 2:3, 3:4, 6:7 and 7:8. There is no difference between males and females.

	fetal age (months)*			
Sex	< 3 3/4	3 3/4 - 5	5 - 6 1/4	> 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	1.13±1.18 n =14	4.17±3.32 n = 18	11.96±6.71 n = 29	36.53±39.53 n = 10
	Group 5	Group 6	Group 7	Group 8
females	1.62±1.06 n = 9	6.60±3.70 n = 17	14.91±7.09 n = 23	30.80±15.86 n = 13

* < 3 3/4 months = < 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length
\> 6 1/4 months = > 22 inches fetal length.

TABLE 11 : Mean and standard deviations of the α-subunit: bLH ratio /pituitary gland in groups 1-8. No statistically significant differences were found between any groups.

Sex	fetal age (months)*			
	$<$3 3/4	3 3/4 - 5	5 - 6 1/4	$>$6 1/4
	Group 1	Group 2	Group 3	Group 4
males	2.05 ± 1.23	1.34 ± 0.95	1.34 ± 0.59	1.12 ± 0.50
	n = 13	n = 18	n = 30	n = 10
	Group 5	Group 6	Group 7	Group 8
females	1.72 ± 1.27	1.56 ± 1.40	1.53 ± 0.86	1.11 ± 0.31
	n = 9	n = 17	n = 24	n = 13

* $<$3 3/4 months = $<$9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
$>$6 1/4 months = $>$22 inches fetal length.

(months) in pituitary glands from male and female fetuses. There was an increase of bFSH in pituitary glands from female fetuses > 5 months gestational age (table 12), as indicated by the significant rise between groups 6 (38.62 $\pm$ 39.96 ng/mg pituitary tissue) and 7 (69.07 $\pm$ 36.10 ng/mg pituitary tissue). This was unique in pituitary glands from female fetuses as shown by the significant increase in the bFSH (ng/mg pituitary tissue) levels in pituitary glands from female fetuses > 5 months gestational age. Groups 3 and 4 (pituitary glands from male fetuses containing 25.56 $\pm$ 48.97 and 38.42 $\pm$ 37.12 ng bFSH/mg pituitary tissue, respectively) had significantly lower bFSH levels than groups 7 and 8 (pituitary glands from female fetuses containing 69.07 $\pm$ 36.10 and 81.43 $\pm$ 43.48 ng bFSH/mg pituitary tissue, respectively). This increase of bFSH in pituitary tissue from female fetuses is further emphasized by the fact that no significant difference was observed between groups 1-4 (pituitary glands from male fetuses.

Total content of bFSH/pituitary gland increased with fetal age in pituitary glands from both male and female fetuses as indicated by the significant increases between groups 3:4 of the males and 6:7, 7:8 of the females (figure 27, table 13). The higher levels found in groups 7 and 8 of females (5.97 $\pm$ 3.26 and 12.84 $\pm$ 7.77 μg bFSH/pituitary gland) compared to groups 3 and 4 of males (1.98 $\pm$ 2.54 and 7.92 $\pm$ 8.55 μg bFSH/pituitary gland),

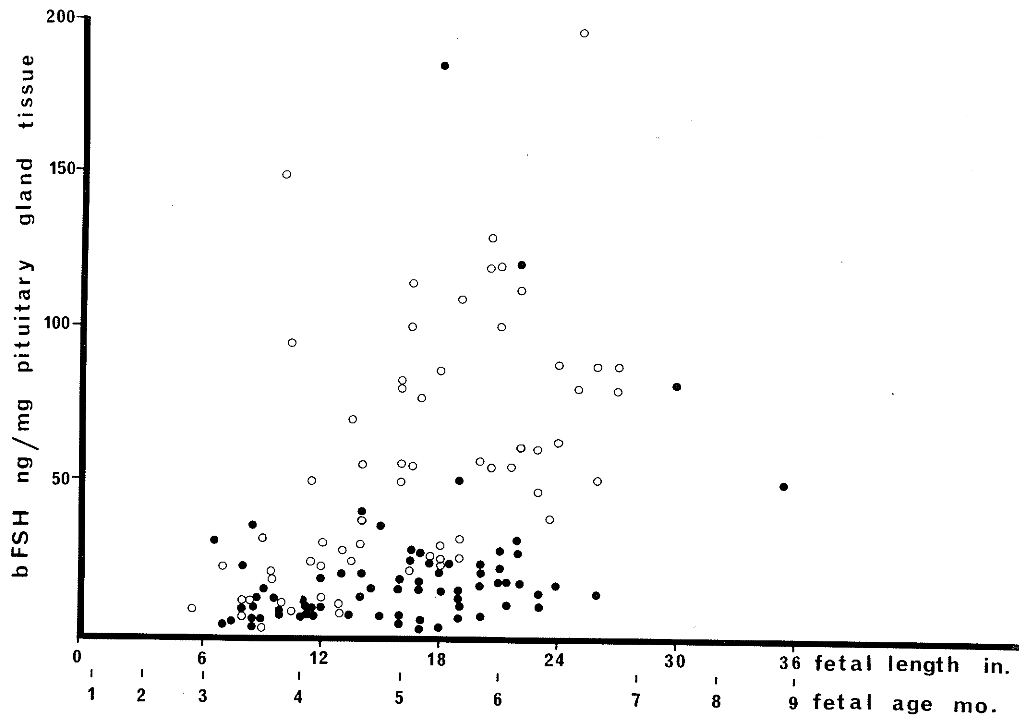

Figure 26 : Relationship between age of fetus (months) and concentration of bFSH (ng/mg) in pituitary tissue as determined by assaying fetal pituitary extracts with the RRA for bFSH. Closed circles (●) represent bFSH concentrations in pituitary glands from male fetuses and open circles (○) represent bFSH concentrations in pituitary glands from female fetuses.

TABLE 12: Means and standard deviations in groups 1-8 of bFSH, ng/mg pituitary tissue. A statistically significant increase ($p < 0.05$) was found between groups 6:7, 3:7 and 4:8.

Sex	fetal age (months)*			
	<3 3/4	3 3/4 -5	5 - 6 1/4	>6 1/4
	Group 1	Group 2	Group 3	Group 4
males	13.08±11.02	13.22±10.10	25.56±48.97	38.42±37.12
	n = 13	n = 18	n = 30	n = 10
	Group 5	Group 6	Group 7	Group 8
females	13.83±9.57	38.62±36.96	69.07±36.10	1.43±43.48
	n = 9	n = 7	n = 23	n = 13

*< 3 3/4 months = <9.9 inches fetal length.
3 3/4 - 5 months = 10.15.9 inches fetal length.
5 - 6 1/4 months - 10-21.9 inches fetal length.
>6 1/4 months = >22 inches fetal length.

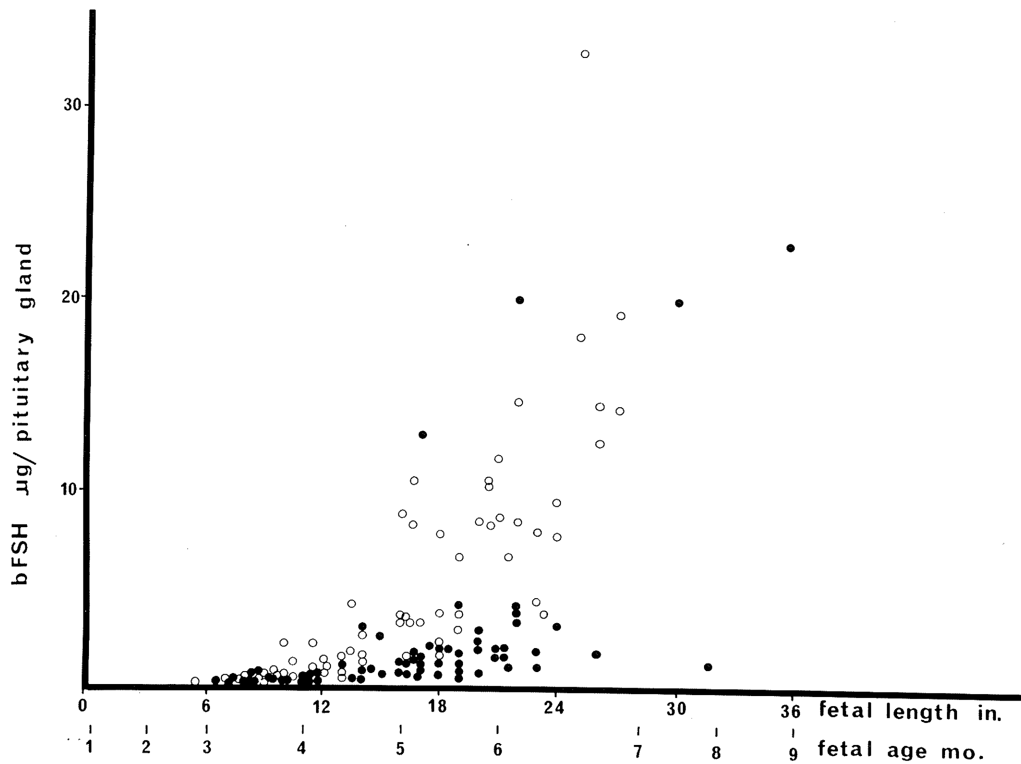

Figure 27 : Relationship between age of fetuses (months) and content of bFSH (µg) per pituitary gland as determined by assaying fetal pituitary extracts with the RRA for bFSH. Closed circles (●) represent bFSH content in pituitary glands from male fetuses and open circles (○) represent bFSH content in pituitary glands from female fetuses.

TABLE 13 : Means and standard deviations of bFSH μg/pituitary, in groups 1-8. Statistically significant increases were found in males between groups 3:4 and in females between groups 6:7 and 7:8. Pituitary glands from female fetuses older than 5 months, contained more bFSH than males from the corresponding age groups as shown by increases between groups 3:7 and 4:8.

	fetal age (months)*			
Sex	$<$3 3/4	3 3/4 - 5	5 - 6 1/4	$>$6 1/4
	Group 1	Group 2	Group 3	Group 4
males	0.13 ± 0.08	0.71 ± 0.77	1.98 ± 2.54	7.92 ± 8.55
	n = 13	n = 18	n = 30	n = 10
	Group 5	Group 6	Group 7	Group 8
females	0.27 ± 0.25	1.54 ± 1.00	5.97 ± 3.26	12.84 ± 7.77
	n = 9	n = 17	n = 23	n = 13

* $<$ 3 3/4 months = $<$ 9.9 inches fetal length.
3 3/4 - 5 months = 10- 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
$>$6 1/4 months = $>$ 22 inches fetal length.

indicated that the increase was more dramatic in pituitary glands from female fetuses.

The ratio of free α-subunit: bFSH in pituitary glands from male fetuses decreased slowly throughout gestation as shown by the decrease in the means and standard deviations between groups 1-4 (table 14). However, the fact that only the decrease between groups 1 and 4 was statistically significant, indicated that the decline of this ratio of free α-subunit: bFSH was gradual with respect to the age of the fetuses. A decrease of the ratio of free α-subunit: bFSH in pituitary glands from female fetuses was found between groups 5 and 6, just prior to the elevation of bFSH levels as shown previously to occur in group 7 (figure 26, table 12). Group 7 (5 - 6 1/4 months female fetuses), which was previously shown to have a higher bFSH content than its corresponding male group (group 3) (figure 27, table 13), was also found to have a signficantly lower free α-subunit: bFSH ratio than group 3 (table 14). No statistically significant difference was found for the ratio of free α-subunit: bFSH (per pituitary gland) between groups 4 (male fetuses 6 1/4 months) and 8 (female fetuses 6 1/4 months).

<u>Bovine Fetal Pituitary Gland Content of TSH</u>

Figure 28 shows the relationship between the concentration of bTSH (ng/mg pituitary tissue) and fetal age (months) in pituitary glands from male and female fetuses.

TABLE 14 : Means and standard deviations of the α-subunit bFSH ratio/pituitary gland in groups 1-8. A statistically significant decrease ($p < 0 .05$) in the α-subunit: bFSH ratio is found in females from groups 5:6. A decrease in this ratio in males is found between groups 1:4. Group 7 has a significantly lower α:bFSH ratio than group 3.

Sex	fetal age (months)*			
	< 3 3/4	3 3/4 - 5	5 - 6 1/4	> 6 1/4
	Group 1	Group 2	Group 3	Group4
males	17.12±11.69 n = 13	13.18±7.75 n = 18	12.20±7.53 n = 30	7.79±6.22 n = 10
	Group 5	Group 6	Group 7	Group 8
females	14.58±13.72 n = 9	7.60±5.66 n = 17	4.78±2.82 n = 23	2.75±0.97 n = 30

* < 3 3/4 months = < 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
> 6 1/4 months = > 22 inches fetal length.

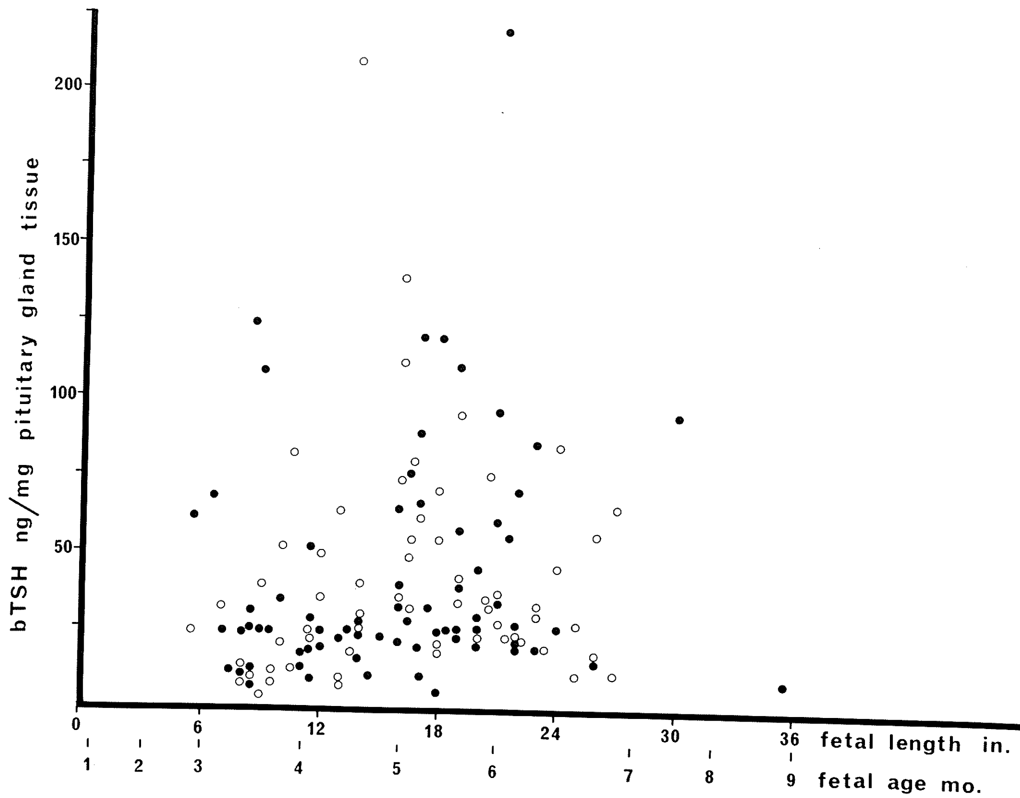

Figure 28 : Relationship between age of fetuses (months) and concentration of bTSH (ng/mg) in pituitary tissue as determined by assaying fetal pituitary extracts with the RRA for bTSH. Closed circles (●) represent bTSH concentrations in pituitary glands from male fetuses and open circles (o) represent bTSH concentrations in pituitary glands from female fetuses.

The means and standard deviations for the eight groups are shown in table 15. There was no significant difference in pituitary concentrations of bTSH between male and female fetuses of corresponding age groups; however, in pituitary glands from male fetuses, the level of bTSH/mg pituitary tissue increased significantly between groups 2 and 3 (20.74 ± 11.20 and 54.21 ± 48.61 bTSH/mg pituitary tissue, respectively). It is interesting to point out that this increase of bTSH in male fetuses at approximately 5 months of age, follows elevated levels of free α-subunit/mg pituitary tissue found in group 1 males (figure 22, table 7).

There was no significant difference in the pituitary content of bTSH (μg) in pituitary glands from male and female fetuses of corresponding age groups (figure 29, table 16); however, significant increases between groups 1:3, 1:4 and 2:3 of males and 5:7, 5:8 and 6:7 of females, indicated an increase in the total fetal pituitary content of bTSH with increasing fetal age.

Table 17 shows that no significant difference in the ratio of free α-subunit: bTSH (per pituitary gland) with respect to the age of the fetuses was found between any groups.

<u>Ratio of Free α Subunit: Total Glycoprotein Hormone per Pituitary Gland</u>

Table 18 shows the means and standard deviations of the ratio of free α-subunit to the total amount of

TABLE 15 : Means and standard deviations of bTSH, ng/mg pituitary tissue, in groups 1-8. A significant increase ($p < 0.05$) was found in males between groups 2:3.

Sex	fetal age (months)*			
	$<$3 3/4	3 3/4 - 5	5 - 6 1/4	$>$6 1/4
	Group 1	Group 2	Group 3	Group 4
males	39.62 $\pm$ 37.09 n = 14	20.74 $\pm$ 11.20 n = 18	54.21 $\pm$ 48.61 n = 30	38.32 $\pm$ 32.35 n = 10
	Group 5	Group 6	Group 7	Group 8
females	17.87 $\pm$ 13.35 n = 9	46.24 $\pm$ 48.42 n = 17	52.97 $\pm$ 31.64 n = 23	33.92 $\pm$ 23.06 n = 13

*$<$3 3/4 months = $<$9.9 inches fetal length.
3 3/4 - 5 months = 10-15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
$>$6 1/4 months = $>$ 22 inches fetal length.

Figure 29 : Relationship between age of fetuses (months) and content of bTSH (µg) per pituitary gland as determined by assaying fetal pituitary extracts with the RRA for bTSH. Closed circles (●) represent bTSH content in pituitary glands from male fetuses and open circles (○) represent bTSH content in pituitary glands from female fetuses.

TABLE 16 : Means and standard deviations of bTSH, μg/pituitary gland in groups 1-8. The significant increases ($p < 0.05$) between groups 1:3, 1:4, 2:3, 5:7, 5:8 and 6:7 indicates that the pituitary content of bTSH increases with fetal age. No difference was found between males and females.

Sex	fetal age (months)*			
	<3 3/4	3 3/4 - 5	5 - 6 1/4	>6 1/4
	Group 1	Group 2	Group 3	Group 4
males	0.36 ± 0.22 n = 14	0.80 ± 0.39 n = 18	4.46 ± 4.13 n = 30	6.36 ± 6.21 n = 10
	Group 5	Group 6	Group 7	Group 8
females	0.27 ± 0.22 n = 8	2.08 ± 2.80 n = 17	4.45 ± 2.56 n = 23	4.80 ± 2.96 n = 13

* < 3 3/4 months = < 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
> 6 1/4 months = > 22 inches fetal length.

TABLE 17 : Means and standard deviations of the α-subunit: bTSH ratio/pituitary gland in groups 1-8. No statistically significant differences were found between any groups.

Sex	fetal age (months)*			
	$<$ 3 3/4	3 3/4 - 5	5 - 6 1/4	$>$ 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	6.47±4.07 n = 13	5.78±3.30 n = 18	4.85±3.12 n = 30	8.28±7.33 n = 10
	Group 5	Group 6	Group 7	Group 8
females	8.43±6.24 n = 9	6.22±4.24 n = 17	5.51±3.89 n = 23	8.75±5.90 n = 13

* $<$ 3 3/4 months = $<$ 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
$>$ 6 1/4 months = $>$ 22 inches fetal length.

TABLE 18 : Means and standard deviations of α-subunit activity: total glycoprotein hormone ratio/pituitary in groups 1-8. A significant decrease ($p < 0.05$) between groups 1:2, 1:3, 1:4, 5:7 and 5:8, indicates an inverse relationship between the ratio of the α-subunit activity: total glycoprotein hormone and the fetal age. No difference between males and females were observed.

	fetal age (months)*			
Sex	$<$ 3 3/4	3 3/4 - 5	5 - 6 1/4	$>$ 6 1/4
	Group 1	Group 2	Group 3	Group 4
males	1.30 ± 0.70	0.90 ± 0.57	0.86 ± 0.33	0.75 ± 0.29
	n = 13	n = 18	n = 30	n = 9
	Group 5	Group 6	Group 7	Group 8
females	1.26 ± 0.86	0.92 ± 0.86	0.79 ± 0.30	0.66 ± 0.15
	n = 10	n = 16	n = 23	n = 14

* $<$ 3 3/4 months = $<$ 9.9 inches fetal length.
3 3/4 - 5 months = 10 - 15.9 inches fetal length.
5 - 6 1/4 months = 10 - 21.9 inches fetal length.
$>$ 6 1/4 months = $>$ 22 inches fetal length.

glycoprotein hormone (per pituitary gland) in fetal pitui-gland as grouped previously according to the age and sex of the fetuses. Significant decreases were observed between groups 1:2, 1:3 and 1:4 of the males fetuses, and between groups 5:7 and 5:8 of the female fetuses. The means and standard deviations decreased with age in both males and females, but the fact that a statistically significant decrease could be found in only two directly adjoining groups (1:2), indicated that the delcine in this ratio is gradual. No difference was found in pituitary glands from male and female fetuses of corresponding age groups.

Absence of Free β-Subunits in Bovine Fetal Pituitary Glands

All of the fetal pituitary extracts were assayed for bFSHβ activity utilizing the RIA for bFSHβ. However, after subtracting out the cross-reactivity in the RIA system due to the presence of intact bFSH in the sample, values obtained for free bFSHβ in all the fetal pituitary extract could not be significantly distinguished from zero.

Similarly, utilizing the RIAs for bLHβ and bTSHβ and subtracting out the cross-reactivities due to the presence of the intact bLH and bTSH in each sample, no free bLHβ and bTSHβ subunits were detected.

Biochemical Characterization of the Free α-Subunit

Molecular Size of the Free α-Subunit as Compared to bLH, bFSH and bTSH

As reported previously (figures 18,19,20), the free α-subunit from bovine fetal pituitary glands was eluted with an identical elution volume to bLHα, bFSHα and bTSHα (as determined by assaying the eluants with the RIA for bFSHα) after gel filtration on a 2x110 cm column of Sephadex G-100 indicating that the free α-subunit from bovine fetal pituitary glands was similar in molecular size to purified bLHα, bFSHα and bTSHα.

Comparison of Cross-Reactivities between bFSHα and Free α-Subunit from Bovine Fetal Pituitary Glands in the RIA for bFSHα

Since the free α-subunit from bovine fetal pituitary glands was detectable by the RIA for bFSHα, and was similar in molecular size to the LHα, FSHα and TSHα standards, the free α-subunit was further characterized for its immunological cross-reactivity as compared to the purified α-subunit preparations.

After gel filtration of pituitary extracts A,B,C, and D on Sephadex G-100, the appropriate fractions containing free α-subunit activity (fractions 134-170) were pooled and lyophillized (figures 19,20). The dried extracts of the free α-subunit were dissolved at a concentration of 10,000 ng/ml in 0.1M NH_4HCO_3 buffer and diluted appropriately in PBS, pH 7.4, containing 0.1% BSA for the RIA for bFSHα.

Figure 30 shows that the free α-subunit cross-reacted in a parallel manner to bFSHα in the RIA for bFSHα indicating that the two were immunologically identical.

Polyacrylamide Gel Electrophoresis of the Free α-Subunit

The lyophilized fraction containing the free α-subunit from pituitary extract D, after gel filtration on Sephadex G-100, was further characterized for its electrophoretic mobility by polyacrylamide gel electrophoresis.

In order to determine the electrophoretic mobilities of the various α-subunit preparations, 20 μg each of bFSHα, bLHα and bTSHα, as well as 50 μg of the lyophilized crude α-subunit fraction from pituitary extract D, were dissolved in 0.012 M Sodium glycinate buffer and applied to the gel. A current of 2m Amp/gel was used until the ion front had travelled a distance of 6 cm. The gel column was sectioned into 5mm gel segments, and the protein was eluted with 0.5% NH_4HCO_3. After appropriate dilutions in PBS, pH 7.4, containing 0.1% BSA, the eluants of each gel segment were assayed with the RIA for bFSHα. In order to visualize the electrophoretic pattern of the proteins, a duplicate set of gel containing 50 μg each of bFSHα, bLHα and bTSHα, as well as 150 μg of the free α-subunit were electrophorized simultaneously. After electrophoresis, the gels were stained for proteins for 1 hour in 1% amido black in 7% acetic acid and destained for 24 hours in 7% acetic acid.

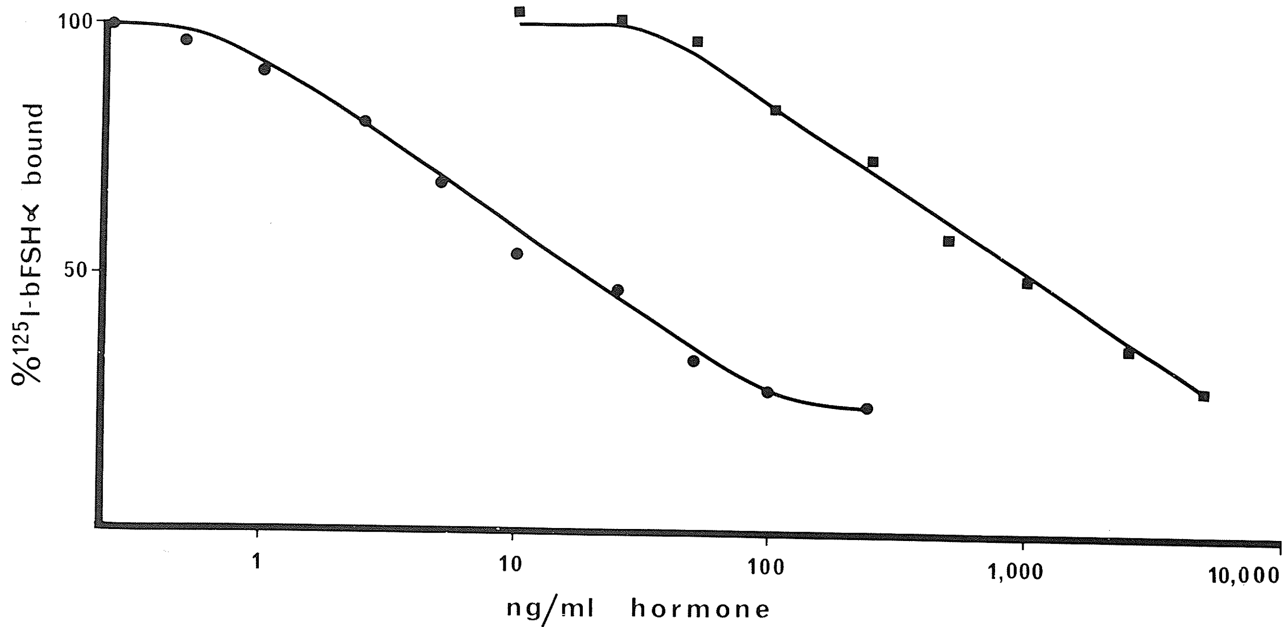

Figure 30 : Inhibition curves of radioimmunoassays for bFSH and the free α-subunit from fetal pituitary extracts. Closed circles (●) = bFSHα and closed squares (■) = free α-subunit in fetal pituitary extracts after gel filtration on Sephadex G-100.

As shown in figure 31, bFSHα and bTSHα each exhibited 1 major peak of activity (as detected by the RIA for bFSHα) in gel segment 4; whereas the major peak of bLHα activity was in gel segment 2. The major peak of free α-subunit activity from fetal pituitary extract D was in gel segment 5.

The stained protein pattern after the electrophoresis showed that protein bands at Rf values corresponded to the peaks of immunologically reactive α-subunit as detected by the RIA for bFSHα. Bovine FSHα exhibited 4 protein bands at Rf values of 0.22, 0.30, 0.38 and 0.45 respectively. There was 2 protein bands for bLHα at Rf values of 0.17 and 0.22, and 5 protein bands for bTSHα at Rf values of 0.17, 0.22, 0.30, 0.38 and 0.45 respectively. There were many protein bands in the gel containing fetal pituitary extract D; however only the two bands at Rf values of 0.30 and 0.38 corresponded with the free α-subunit activity as detected by RIA.

The elctrophoretic mobilities of the different α-subunit preparations varied slightly even though they were relatively similar.

Recombination of Subunits

Since the free α-subunit from bovine fetal pituitary glands was similar to bLHα, bFSHα and bTSHα in molecular size after gel filtration on Sephadex G-100, immunological cross-reactivity in the RIA for bFSHα and electrophoretic

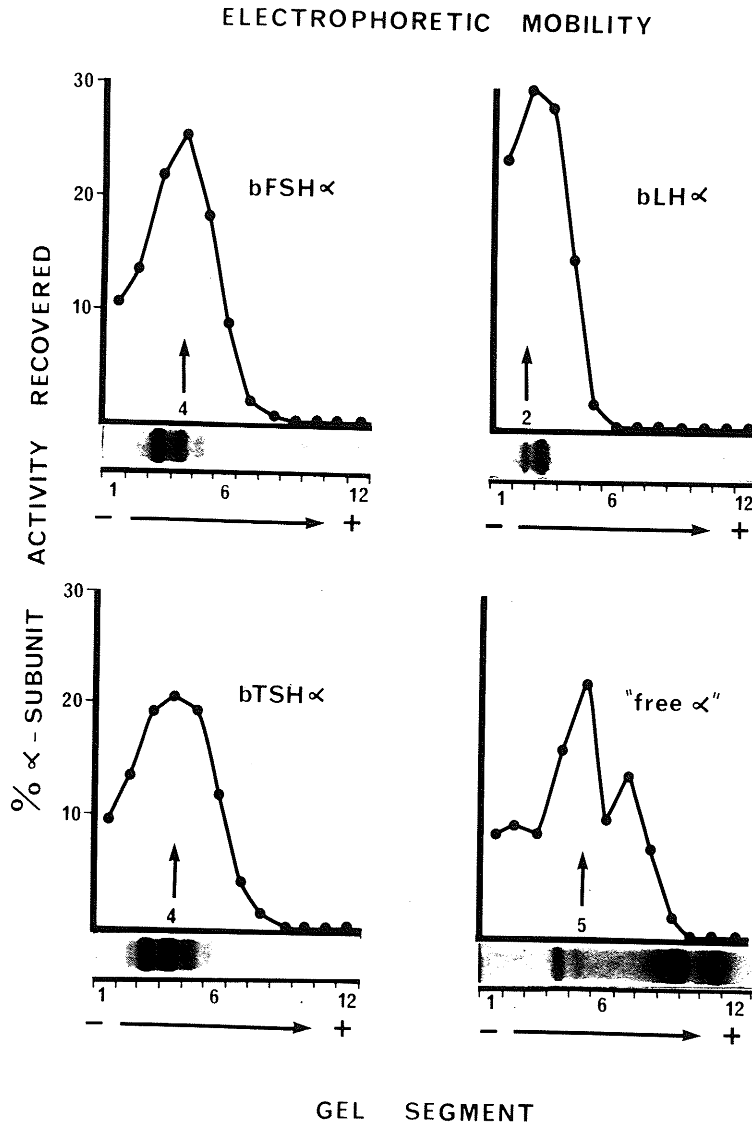

Figure 31 : Electrophoretic mobilities of bFSHα, bLHα, bTSHα and the free α-subunit in fetal pituitary extracts after gel filtration on Sephadex G-100.

mobility after polyacrylamide gel elctrophoresis, an attempt was made to recombine this free α-subunit with bFSHβ to regenerate bFSH activity.

The lyophilized fractions containing the free α-subunit (after gel filtration on Sephadex G-100) from pituitary extracts B and C were incubated with an equal amount of purified bFSHβ. The amount of bFSH activity regenerated from recombinations of the subunits was determined by the RRA for bFSH. The results of this study are summarized in table 19.

Only bFSHα and bFSHβ were recombined significantly regenerating 57.5% bFSH activity. The FSH activity detected in the samples containing bFSHβ and the free α-subunit from pituitary extracts B or D was similar to the bFSH activity detected in the incubation medium containing either the bFSHα or bFSHβ controls. The free α-subunit found in bovine fetal pituitary glands differed from the purified bFSHα preparation in that it did not recombine with the β-subunit to regenerate biologically active intact hormone.

TABLE 19 : Recombination of subunits. The RRA for bFSH was used to determine bFSH activity present in each sample after the 16 hour incubation period. The % bFSH activity present was calculated as the

$$\frac{\text{bFSH Activity Recovered (Mg)}}{\text{Total hormone present in incubation sample (mg)}} \times 100$$

Incubation Sample	bFSH Activity Recovered (μg)	%bFSH Activity Present
10 μg bFSH	8.2	82
10 μg bFSHα	0.08	0.8
10 μg bFSHβ	0.072	0.72
10 μg bFSHα + 10 μg bFSHβ	11.5	57.5%
5.1 mg α subunit activity from fetal pituitary extract B+ 5 mg bFSHβ	0.11	1.1
10.48 mg α subunit activity from fetal pituitary extract D+ 10 mg bFSHβ	0.5	2.5

DISCUSSION

The Radioreceptor Assay for bTSH

The radioreceptor assay developed for bTSH has been shown to be sensitive and specific (figure 5). In comparison to other RRA systems reported for TSH, this assay has the advantage of simplicity as it does not require either sucrose density gradient centrifugation for the preparation of receptor membranes as do the assay systems reported by Winand and Kohn (53) and Aiginger and Talley (65), and it does not require a cell culture system as does the assay reported by Lissitzky et al. (66). In addition, this RRA for TSH is more sensitive than all of the previously mentioned RRA systems. Because of the sensitivity of the assay and the relatively simple procedure in preparing large amounts of membrane receptor, this RRA appears to be the most convenient one reported to date for monitoring a large number of samples.

Studies on the application of this RRA have shown that bTSH and rTSH of similar biological potencies interact on an equal weight basis with the porcine thyroid membrane receptor (figure 6). In addition, the observation that TSH activity in pituitary extracts from dog, rabbit, rat, guinea pig, turkey and chicken interacted with the porcine thyroid membrane receptor

in a parallel manner with the bTSH standard (figure 7), indicated that there might be an inter-species similarity in the interaction of the TSH molecule with its membrane receptor. Further, it has been shown by utilizing this RRA that although the TSH activity from different species exhibited slight variations in their electrophoretic mobilities (figure 9), the size of the TSH molecule from different species was similar.

Because the TSH activity in pituitary extracts from cow, dog, rabbit, rat, guinea pig, turkey and chicken can be monitored by the radioreceptor assay developed, this assay system can be a valuable tool for future biochemical and physiological studies of TSH activity in different species.

The Radioimmunoassays for bFSH, bFSHα and bFSHβ

The RIA system for bFSH has been shown to be sensitive and specific (figure 12). This is the first homologous radioimmunoasssy developed for bFSH, and has been employed in these studies of bovine fetal pituitary FSH levels and also in the studies of cattle serum FSH levels (67). This RIA will be a valuable tool for further studies to understand the role of FSH under various physiological conditions in cattle. To date, this area of reproductive endocrinology of cattle has been almost untouched due to the lack of a specific RIA for bFSH.

The RIA system for bFSHβ is a sensitive means for physiological and biochemical studies of the specific β-subunit. The cross-reactivity of intact bFSH in this assay system (figure 14) makes this RIA system useful for monitoring intact bFSH activity even in the presence of bLH and bTSH but in the absence of free bFSHβ.

The RIA system for bFSHα is sensitive, and because of its ability to monitor purified preparations of bFSHα, bLHα and bTSHα identically (figure 13), makes it a valuable tool for futur biochemical and physiological studies of the common α-subunit of these glycoprotein hormones.

Free α Subunit in Bovine Fetal Pituitary Glands: Relationship to Glycoprotein Hormones

Kaplan and Grumbach (41) and Hagen and McNeilly (35) have reported the presence of free α-subunit in human fetal pituitary glands. In the present study, free α-subunit has been shown to be present in bovine fetal pituitary glands of different gestational stages (figure 23) indicating that similar patterns of ontogenesis for pituitary glycoprotein hormones might occur in humans and in cattle. Furthermore, the presence of free FSHβ, LHβ and TSHβ in pituitary glands have not been reported in the literature, and in the present studies, free β subunits have not been detected in any of the fetal pituitary glands.

As a result of the experimental evidence in humans that release of free pituitary α-subunit occurred prior

to release of LH after LHRH administration (34), Hagen and McNeilly (35) proposed that in the pituitary gland the common α-subunit is synthesized first followed by the synthesis of the β subunits under hypothalamic control, thus leading to the production of intact hormones. The results of the present study on bovine pituitary glands tend to support that proposal as changes of free α-subunit levels were detected in all cases prior to elevations of intact glycoprotein hormones (figures 22,23).

In 1970, Fukuchi etal.(27) reported that the TSH content in human fetal pituitaries expressed in terms of μg per fetal body weight, was maximal at 12-17 weeks of gestation. In the cattle, pituitary concentrations of TSH (ng/mg pituitary tissue) were found to increase in pituitary glands from male fetuses at approximately 5 months gestational age (table 15). Pituitary glands from male fetuses less than $3^3/4$ months gestational age contained significantly more free α-subunit than pituitary glands from female fetuses of the corresponding age group (table 7), indicating, a possible correlation between increases of free α-subunit synthesis prior to elevations of TSH in the bovine fetal pituitary glands of males.

On the other hand, pituitary concentrations of bFSH increased significantly in pituitary glands from

female fetuses older than 5 months gestational age, but not in pituitary glands from male fetuses (figures 26, 27; tables 12,13). Similarly, in humans, FSH levels in pituitary glands from female fetuses have been reported to be higher at a fetal age of 25-29 weeks as compared to fetuses aged 10-14 weeks (37,41). Kaplan and Grumbach (37) and Faiman et al. (39) proposed that this male-female difference in FSH levels was due to elevated testosterone levels found in the serum of male fetuses, aged 12-20 weeks, resulting from differentiation of the genital tract in males and the development of a functional hypothalamic pituitary-gonadal feedback system during fetal life. To date, there have been no reports on the serum levels of testosterone in bovine fetuses; however, the increase in bFSH levels found in pituitary glands from female fetuses occurs at a similar time (5 months gestational age) as the testes descend into the scrotum in male fetuses (68), thus indirectly correlating the bFSH burst found in pituitary glands from female fetuses. These observations in bovine fetuses are in good agreement with the suggested hypothesis derived from the human studies for the development if the pituitary-gonadal feedback system in male fetuses at the gestational age of 5 months (37,39).

Although no elevated levels of free α-subunit were found in pituitary glands from female fetuses prior to the elevated bFSH levels found after 5 months gestational age, the ratio of the free α-subunit: bFSH (per pituitary gland)

was significantly lower in pituitaries from female fetuses at 5-6¼ months gestational age as compared to pituitary glands from female fetuses of the corresponding age group (table 14). The difference of this ratio between males and females indicated that the appearance of free α-subunit in bovine fetal pituitary glands was not coincident with the appearance of the intact hormone, but suggested that the free α-subunit in the female fetal pituitary glands might be used in the generation of intact bFSH. This suggestion is further emphasized by the fact that the decreased value for the ratio of free α-subunit: bFSH found in pituitary glands from female fetuses was only transient, as the value for his ratio was again similar in pituitaries from male and female fetuses greater than 6¼ months gestational age (table 14) even though the amount of bFSH remained significantly higher in pituitary glands from female fetuses (figures 26,27; tables 12,13).

In this study, the values obtained for the concentrations of bLH (ng/mg) in fetal pituitary tissue (figure 24; table 9) were similar to those reported by Oxender, Convey and Hafs (26) in their study of LH levels in bovine fetal pituitary tissue from fetuses of 90, 180 and 260 days gestation. No difference in the pituitary LH content was found between the sexes of the fetuses (figure 25,table 10). Data of the present study are in good agreement with the reports published by Oxender, Convey and Hafs (26) in their work with bovine fetal pituitaries, and Hagen and McNeilly

in their work with human fetal pituitaries; however, the present findings disagreed with the reports by Kaplan and Grumbach (37,41) who found that fetal pituitary concentrations of LH were higher in females than males after the 25th week of gestational age. The observation that no significant difference was observed for the ratio of free α -subunit:bLH between pituitary glands of male and female fetuses throughout gestation (table 11), tended to support the findings that the bLH content of pituitary glands from male and female fetuses was similar.

The total amount of all of the glycoprotein hormones, as well as the amount of the free α-subunit in fetal pituitary glands increased proportionately with fetal age (figures 23,25,27,29;tables 8,10,13,16). The finding that the ratio of the free α-subunit: total amount of glycoprotein hormones (per pituitary gland) decreased gradually throughout gestation (table 18), and that this ratio was similar between male and female fetuses throughout gestation in spite of the existance of some differences in the pituitary content of the different glycoprotein hormones between male and female fetuses at various stages of gestation, indicated the possibility of correlation for the production of the free α-subunit for the generation of the different glycoprotein hormones in the bovine fetal pituitary gland throughout gestation.

Characterization of the Free α-subunit

The free α-subunit in bovine fetal pituitary extracts has been characterized in comparision with the purified preparations of bFSHα, bLHα and bTSHα isolated by dissociating the biologically active intact hormones. All showed a similarity in their elution volumes after gel filtration on Sephadex G-100 (figures 18,19,20) indicating that the free α-subunit found *in vivo* was similar in molecular size to purfied preparations of bFSHα,bLHα and bTSHα. Immunologically, the free α-subunit cross-reacted in a parallel manner to the bovine α-subunit standards (figure 30) indicating that they might be structurally very similar or identical.

The electrophoretic mobility of the free α-subunit was similar but not identical to that of either LHα, FSHα or TSHα (figure 31). This could be accounted by the fact that the electrophoretic mobilities of purified preparations of bLHα, bFSHα and bTSHα varied slightly (figure 31) even though their primary amino acid sequences are identical (4,5,6,7,8,9). Variations in electrophoretic mobilities might be a result of differences in the composition of the carbohydrate residue in the different subunit preparations as reported by Maghuin-Rogister el al.(13).

Weintraub et al. (23) reported that an attempt to recombine free HCGα subunit,purified from cell cultures, with HCGβ failed to regenerate intact HCG activity.

Similarly, in the present study, the free α-subunit from bovine fetal pituitary glands failed to recombine with bFSHβ to regenerate bFSH activity (table 19), indicating the existance of some yet unknown structural or functional differences between the free α-subunit in the pituitary tissue and the FSHα subunit resulting from the dissociation of the intact bFSH molecule. The exact structural and functional relationships of this free α-subunits to bFSHα, bLHα and bTSHα remain to be elucidated with further physiological and biochemical studies upon the purification of this free α-subunit component.

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