July 29, 1968

To the Graduate Council:

I am submitting herewith a dissertation written by Pin-Kwen Chung entitled "Radioimmunoassay for Bovine Luteinizing Hormone (BLH)." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

R. L. Murphree
Major Professor

We have read this dissertation
and recommend its acceptance:

Raymond G Cragle

Sam L Hansard

Robert H. Feinberg

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
Graduate Studies and Research

RADIOIMMUNOASSAY FOR BOVINE LUTEINIZING HORMONE (BLH)

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Pin-Kwen Chung

August 1968

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. R. L. Murphree for his guidance, encouragement and help throughout this study; to Dr. Wen-Kwang Yang, Biology Division, Oak Ridge National Laboratory for his assistance; to Dr. W. D. Odell, Endocrinology Division, Harbor General Hospital, California, for his instructions; and to Miss M. L. Swatzell for her careful reading of the manuscript.

Appreciation is expressed to committee members, Drs. R. G. Cragle, R. H. Feinberg, R. R. Shrode and S. L. Hansard for their advice and comments pertaining to the dissertation.

The author also wishes to express gratitude to Dr. Nathan S. Hall, Laboratory Director, for his kindness in awarding him an assistantship and making possible the opportunity to conduct this research problem at The University of Tennessee-Atomic Energy Commission Agricultural Research Laboratory. Thanks is granted to the animal caretakers and personnel in the laboratory for their help and co-operation.

Special thanks go to his father, wife and daughters for their encouragement, patience and profound understanding during this period of graduate study.

ABSTRACT

Recent progress in immunological techniques has revealed the possibility for more sensitive methods for assaying protein hormones. A radioimmunoassay technique has been developed for the detection and direct measurement of physiological levels of human pituitary luteinizing hormone in blood plasma. The radioimmunoassay is based on the competition between ^{131}I-labeled hormone and unlabeled hormone for antibodies to the hormone. The separation of free hormone from antibody-bound hormone is achieved by precipitation using antibody to γ-globulin of an animal in which the antihormonal serum was produced.

This study was initiated to develop the radioimmunoassay technique which could be used to measure physiological levels of bovine LH directly from blood without concentration or purification. The assay was used to measure the variation of luteinizing hormone in the blood of four cows during the estrual cycle. The average levels of the hormone was less than 1.5 mμg. equivalents of the standard (NIH-LH-B5) per ml. of serum during diestrus but increased 10 to 20 fold during estrus. The concentration of the hormone in the blood increased sharply in the early stages of estrus and decreased rapidly in the late stage of estrus. The results indicated that the procedures can be used to measure the luteinizing hormone activity in 1 ml. of blood.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

CHAPTER I

INTRODUCTION

Recent progress in immunological techniques has revealed the possibility for more sensitive assay methods for hormones which are protein in nature. Radioimmunochemical techniques have been well established for the detection and direct measurement of physiological levels of human pituitary luteinizing hormone (LH) and human chorionic gonadotropic hormone (HCG) in unconcentrated blood plasma or urine (Wilde, Orr and Bagshawe, 1965, 1967; Odell, Ross and Rayford, 1967a). The radioimmunoassay technique is based on the quantitative competition between the radioisotope labeled hormone and unlabeled hormone for antibodies. This method demonstrated that ^{131}I-LH, bound in vitro by anti-LH serum, can be quantitatively separated by chromatoelectrophoresis or precipitated by an antibody to γ-globulin of an animal in which the antihormonal serum was produced.

It was the purpose of this study to develop a radioimmunoassay technique which could be used to detect physiological levels of bovine LH directly from serum without concentration or purification of the blood sample. This technique was also used to measure the change in the LH level of serum in the normal estrous cycle of cows.

CHAPTER II

LITERATURE REVIEW

Historical background. Immunological studies with protein hormones were begun in the mid-1930's and were reviewed in detail (Collip, 1935; Thompson, 1941; Leathem, 1949). They often dealt with the subject of "antihormones". Collip and Anderson (1934) reported that, after continued administration of thyrotropin (TSH) to rats, the animals became refractory to further stimulation and their serum inhibited thyrotropin activity in untreated rats. The inhibitory effect was said to be due to antihormones. Thompson (1941), in a review, commented that the early literature seems to be confused by the number of hormones, the variation of sources, the wide variety of test animals and the number of different approaches used in the study of the problem.

In most of the earlier studies, relatively crude hormone preparations were employed and attention was usually focused upon the ability of the antisera to neutralize the biological activity of the exogenously administered or endogenously secreted hormone. Because of the relatively crude nature of the hormone preparations employed in the majority of the earlier investigations, the specificity of the observed effects has been frequently questioned. The increasing availability of highly purified hormones and the introduction of new immunological methods have resulted in translating these earlier observations into modern concepts of antigen-antibody reactions.

Potent antisera were obtained by injection of highly purified bovine growth hormone (BGH) and human growth hormone (HGH) in rabbits with the addition of suitable adjuvants, and the specificity of the reaction between growth hormone and its antiserum was demonstrated. The possibility of utilizing immunological methods for the detection and measurement of pituitary growth hormone was suggested (Hayashida and Li, 1958a, b). The technique of hemagglutination-inhibition (Boyden, 1951) was first adopted by Read and Bryan (1960) to measure the level of growth hormone in human sera. But due to the relatively low sensitivity, growth hormone could not be detected in sera of normal adults by this procedure.

The methods developed during the study of insulin metabolism by Berson et al., (1956) permitted quantitative evaluation of the rate of ^{131}I-insulin metabolism and stimulated interest in the feasibility of assaying many other proteins. A direct measurement of HGH in plasma was reported by Glick et al., (1963). They modified the technique developed in the same laboratory by Yalow and Berson (1960) for assaying HGH in plasma. The isotopic method developed by Hunter and Greenwood (1962) of labeling HGH for the radioimmunoassay of HGH is now in common use and has been applied to the assay of many other pituitary hormones. Subsequently, assays for adrenocorticotropin (Yalow et al., 1964), thyrotropin (Odell, Wilber and Paul, 1965), luteinizing hormone (Midgley and Jaffe, 1966), follicle-stimulating hormone (Midgley, 1967), human chorionic gonadotropic hormone (Bagshawe, Wilde and Orr, 1966) in the human have been reported.

Principles of radioimmunoassay. The fundamental principles of the radioimmunoassay employing immunological methods are based on an antigen-antibody reaction. The original assay for insulin (Berson et al., 1956), formed the basis for subsequent radioimmunoassay methods. The radioimmunoassay of insulin described by Yalow and Berson (1960) was based on the reaction between ^{131}I-labeled insulin and insulin antibody. Antibodies to the hormone are detectable by their ability to bind ^{131}I-labeled hormone and this binding is competitively and quantitatively inhibited by unlabeled hormone. The competitive nature (Berson and Yalow, 1964) of the reaction is shown in Figure 1.

$$^{131}\text{I-Hormone} + \text{Hormone Antibody} \rightleftharpoons \text{Antibody-Bound Hormone}^{-131}\text{I}$$

$$+$$

$$\text{Hormone} \rightleftharpoons \text{Antibody-Bound Hormone}$$

Figure 1. Diagram showing competition between labeled hormone and unlabeled hormone for binding to a specific antibody.

Unlabeled hormone competes with ^{131}I-labeled hormone for the antibody, thereby decreasing the percentage of ^{131}I-hormone bound to the antibody. The ratio of bound ^{131}I-hormone to unbound ^{131}I-hormone decreases progressively with increase in unlabeled hormone concentration. A standard curve can be constructed in relation to reference hormone concentrations. The hormone concentration in an unknown sample is then determined by comparison with the standard curve for a reference hormone preparation.

The two techniques commonly used to separate antibody-bound hormone-^{131}I and free hormone-^{131}I from the reaction mixture are chromatoelectrophoresis and double antibody system. These two techniques will be described below.

Methods of Radioimmunoassay

The two methods in current use depend upon the same basic principles. If unlabeled hormone or serum containing the endogenous hormone is added to the specific antiserum to a hormone, it inhibits the ability of that antiserum to bind a labeled hormone. The antibody-bound hormone can be separated in the two following ways:

Chromatoelectrophoresis. In assaying insulin in human plasma as described by Yalow and Berson (1960), a series of standard preparations of the reference hormone (unlabeled) or of the sample under assay are mixed with a constant amount of labeled hormone and antiserum to the hormone. When the reaction has reached equilibrium, the mixture is subjected to chromatoelectrophoresis on filter paper. Any antibody-bound hormone moves with β-γ-globulins while free hormone remains fixed at the origin. A standard curve is constructed to relate the ratio of bound hormone: free hormone (B/F) to the amount of unlabeled reference hormone added. From this standard curve and the ratio found with the sample, the hormone content of the sample can be estimated.

Since being described by Yalow and Berson (1960), this method has been used to assay growth hormone (Glick et al., 1963), thyrotropin (Utiger 1964) and parathyroid hormone (Berson et al., 1963) in human plasma.

Double antibody system. Skom and Talmage (1958) first described the double antibody technique for assaying insulin. Antiserum was obtained by injecting human serum γ-globulin into rabbits and was used for the precipitation of ^{131}I-labeled insulin and globulin in the serum of insulin-treated patients (containing anti-insulin antibodies). The relative proportions of labeled hormone in the precipitate (bound insulin) and in the supernatant solution (free insulin) were used to evaluate the insulin-binding capacity of serum. Their method has been used to assay growth hormone (Utiger et al. 1962), insulin (Morgan and Lazarow, 1963), adreno-corticotropin (Felber, 1963), human chorionic gonadotropin (Odell, Ross and Rayford, 1966), luteinizing hormone (Odell, Ross and Rayford, 1967a), and follicle-stimulating hormone (Midgley, 1967) in human plasma.

The procedure for the double antibody technique is as follows. The specific antiserum is mixed with constant amounts of labeled hormone and a series of reference preparations or unknown samples as described above in chromatoelectrophoresis. After equilibration of the reaction mixture, the antibody-bound hormone is precipitated by the addition of a specific anti-globulin serum and free hormone remains in the super-natant solution. From the ratios of bound to free hormone found with varying quantities of the unlabeled reference hormone, the hormone con-tent corresponding to that of the unknown sample can be determined.

The technique of the double antibody system was adopted for this study i.e., assaying LH in bovine serum. It has advantages which render it more suitable for routine assay and does not require the use of special laboratory equipment.

Luteinizing Hormone Concentration in Human and Bovine Blood

Human. Paul and Odell (1964) developed a radioimmunological technique for assaying HCG. In this system, the presence of the hormone was detected by its competition with radioiodinated HCG for HCG antibodies produced in rabbits. The immune complex was then precipitated with sheep anti-rabbit γ-globulin serum. Later, Odell, Ross and Rayford (1966, 1967) developed a radioimmunoassay for human LH based on cross-reaction between human LH and HCG. Antiserum was developed against HCG and highly purified LH was labeled with ^{131}I. The separation of antibody-bound LH-^{131}I from free LH-^{131}I was accomplished by a double antibody technique. In this system, they were able to measure the minimal amount of 0.15 mμg. (1.2 mμ. in terms of Second International Reference Preparation of Human Menopausal Gonadotropin - 2nd IRP-HMG) of human LH per ml. of plasma.

Radioimmunoassay for HCG and human LH based on a double antibody technique was also described by Wilde, Orr and Bagshawe (1965; 1967). Aggregated HCG antibody complex was separated from the free hormone by filtration on an Oxoid cellulose acetate membrane instead of by centrifugation as described by Odell, Ross and Rayford (1967). A concentration as low as 0.07 I.U. HCG per ml. of urine or serum was detected. By reducing the concentration of the reactants (anti-HCG serum, labeled HCG standard or unknown solution) and increasing the duration of incubation time, 0.001 I.U. per ml. could be measured.

Bovine. Studies of LH in bovine blood employing the radioimmunoassay have not been reported. However, there are two reports (Anderson and McShan, 1966; Varian, Henricks and Melampy, 1967) on the LH level in bovine plasma using the technique of ovarian ascorbic acid depletion (OAAD) described by Parlow (1961). In the study by Anderson and McShan (1966), blood samples were collected from lactating cows which had shown at least one previous estrus following parturition. The LH was concentrated by acetone fractionation and the activity of LH was measured by the OAAD assay. The level of LH ranged from 25 µg. equivalents of NIH-LH-S1 per 100 ml. of plasma to less than 1 µg. Concentration of LH in the blood plasma rose rapidly 6 to 17 hours prior to ovulation but was low during diestrus. They indicated that this procedure could be used to measure the LH activity in 100 ml. of whole blood.

The LH concentration in peripheral plasma of Angus heifers has also been reported (Varian, Henricks and Melampy, 1967). The LH in the blood was concentrated by ethanol-acetone precipitation procedures. Six estrous cycles from four heifers were examined. An LH peak as high as 35 µg. equivalents of NIH-LH-B5 per 100 ml. of plasma was found about 4 hours after the onset of estrus. This peak was maintained for less than 3 hours and then dropped to 2 to 5 µg. per 100 ml. of plasma in late estrus. By day 7 after the estrus, LH again increased about four fold to 10 to 20 µg. per 100 ml. of plasma and remained high until the late luteal phase. Ovulation occurred 24 to 32 hours after the increase in LH.

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

Bovine luteinizing hormone (BLH). Bovine LH was obtained from the National Institutes of Health as NIH-LH-B5. The activity of the NIH-LH-B5 has been determined by the ovarian ascorbic acid depletion assay (Parlow, 1961), and the potency of this hormone obtained from eight replicate assays was 0.87 NIH-LH-S1 units per mg. which corresponds to 1338 I.U. per mg. in terms of 2nd IRP-HMG.

Rabbit anti-BLH serum. Rabbit anti-BLH serum was raised to NIH-LH-B5 in mature Dutch female rabbits. One and one-half mg. of LH were dissolved in 2 ml. of physiological saline (0.85% NaCl) and this solution was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). A total of 4 ml. of this mixture was given subcutaneously (1 ml. in each leg) to each rabbit; four such injections were administered a week apart. For the fifth and sixth injections, 2 mg. of BLH were dissolved in 2 ml. of saline and administered intraperitoneally on subsequent weeks. The animals were bled from a marginal ear vein 10 days after the last injection. If more blood was required, a booster injection in saline was given 10 days before collection. Anti-BLH serum was separated by centrifugation at 500 g. for 20 minutes and 0.1% (V/W) of sodium azide was added as a

preservative. Purity of the anti-BLH serum was examined by the techniques of double gel-diffusion and immunoelectrophoresis (Campbell et al., 1964); the serum was diluted 1:50, distributed into 1 ml. fractions in small vials and stored at -25°C.

Rabbit γ-globulin. Rabbit γ-globulin was precipitated from rabbit serum by the addition of one-third of a saturated ammonium sulfate solution. The ammonium sulfate was then removed by desalting on a Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column. Further purification was made on a diethylaminoethyl cellulose (DEAE) column. Both the Sephadex G-25 and DEAE columns were previously equilibrated with 0.02 M phosphate buffer, pH 6.6.

Sheep anti-rabbit γ-globulin serum (second antibody). Sheep anti-rabbit γ-globulin serum was prepared by dissolving 30 mg. of rabbit γ-globulin (prepared as described above) in 3 ml. of saline and mixing with an equal volume of complete Freund's adjuvant. A total of 6 ml. of this mixture was injected subcutaneously (1 to 2 ml. in each leg) in a sheep every other week for three weeks. The fourth injection was administered intraperitoneally (50 mg. of rabbit γ-globulin dissolved in 5 ml. of saline). Ten days after the last injection, the animal was bled from the jugular vein and the serum was obtained by centrifuging at 500 g. for 20 minutes. The serum was then mixed with 0.1% (V/W) of sodium azide, dispensed in 2 ml. portions in small vials and stored at -25°C.

Diluent. The diluent for antiserum, labeled BLH, and standard solutions was a 0.04 M solution of NaCl in 0.04 M phosphate buffer, pH 7.7, containing 0.1% (V/W) of crystallized-lyophilized ovalbumin and 0.1% (V/W) of sodium azide.

Bovine serum. Blood was collected from four nonpregnant cows (two Herefords, one Holstein and one Angus-Hereford cross). Blood was obtained from the jugular vein at first detection of estrus and at 12-hour intervals during estrus, then at 2 to 3 day intervals during diestrus. Collections were continued until 1 day after the next estrous period. The blood was kept at room temperature until clotted, and then the serum was separated by centrifugation and stored at -25°C. with 0.1% (V/W) of sodium azide added as a preservative. The sera were stored for 4 to 7 months before assays were conducted.

II. METHODS

Absorption of antiserum. Rabbit anti-BLH serum was absorbed by the addition of normal bovine serum. The mixture was incubated at 37°C. for 20 minutes and kept at 4°C. overnight. Then the mixture was centrifuged at 500 g. for 15 minutes; the purity of antiserum in the supernatant was examined by the techniques of double gel-diffusion and immunoelectrophoresis before use for assaying LH.

Gel filtration of BLH. The Sephadex G-100 column (1.5 cm. x 55 cm.) was prepared according to the instructions furnished by the company. The

filtration was conducted at 4°C. with a flow rate of 30 ml. per hour and fraction size of 2.6 ml. The column was previously equilibrated with 0.05 M ammonium bicarbonate (NH_4HCO_3) buffer, pH 8.0. Ten mg. of NIH-LH-B5 were dissolved in 1 ml. of the same solvent buffer and transferred to the column. Elutes (Figure 2) under these two peaks were combined into six fractions which then were evaluated by double gel-diffusion and immunoelectrophoresis. Fraction 1 was verified as a serum protein contaminant in the NIH-LH-B5 preparation and Fraction 5 was verified as a BLH fraction. This latter fraction was also further confirmed as a BLH fraction by use of anti-BLH serum obtained from Dr. W. D. Odell (Univ. of Calif., Los Angeles, Calif.). Fraction 5 was used for the preparation of the ^{131}I-labeled BLH.

Double gel-diffusion. The Ouchterlony double gel-diffusion method (Campbell et al., 1964) was modified to evaluate the purity of the antigen-antibody system of rabbit anti-BLH serum. Phosphate buffered saline, 0.05 M, pH 7.2, containing 0.85% of Ionagar No. 2 (Oxoid, London, England) and 0.1% of sodium azide in the buffer solution was boiled until the agar was completely dissolved. Then the solution was poured into a 60 mm. Petri dish and allowed to harden. Wells with a diameter of 5 mm. were cut and the agar removed by suction. The wells were sealed with agar solution by using a dropper and were then filled with appropriate antiserum and antigen solutions. The dish was kept in a humid chamber at room temperature for 48 hours and the lines of precipitate were stained with 0.6% of aniline blue black (Allied Chemical and Dye Corporation,

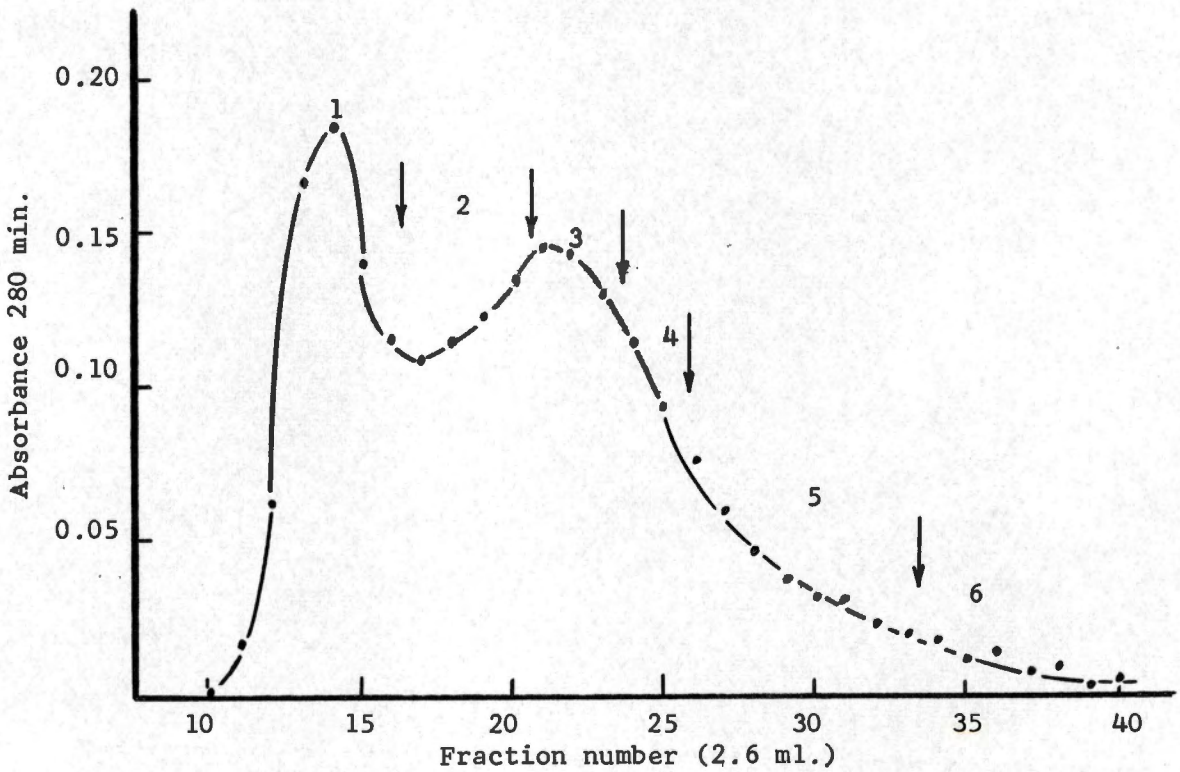

Figure 2. Gel filtration of BLH on Sephadex G-100 column. The NIH-LH-B5 (10 mg.) was dissolved in 1.0 ml. of 0.05 M ammonium bicarbonate, pH 8.0 and transferred to a Sephadex G-100 (1.5 cm. x 55 cm.) which was previously equilibrated with the same buffer, combined number 5 fraction was used as BLH for iodination.

N. Y.) dissolved in methanol, acetic acid and distilled water (45:10:45). Precipitation lines (Figure 3) developed when unabsorbed anti-BLH serum was reacted with bovine serum, NIH-LH-B5 and various fractions of BLH from the Sephadex G-100 column (Figure 2). After the absorption of anti-BLH serum with bovine serum, positive precipitin reactions occurred only when absorbed anti-BLH serum reacted with NIH-LH-B5 and Fractions 4 and 5 from gel filtration of BLH (Figure 2). No precipitin reactions occurred with bovine serum.

Immunoelectrophoresis. The immunoelectrophoresis technique (Campbell et al., 1964) was used for examining the purity and identifying the individual components of rabbit anti-BLH serum. The various BLH and its Sephadex G-100 column fractions (Figure 2) were separated by immunoelectrophoresis (LKB, Stockholm, Sweden) for 70 minutes at 250 volts and 26 amperes on agar slides into a series of spots in the electrophoretic chambers containing barbital buffer, pH 8.2, u = 0.05. Troughs were then cut in the agar parallel with the line of migration of the BLH and its fractions from the Sephadex column. The troughs were filled with absorbed and unabsorbed anti-BLH sera and then kept in the humid chamber at room temperature for 48 hours. Lines of precipitate (Figure 4) were stained with 0.6% of aniline blue black dye as described in the previous section. Results shown in Figure 4 were similar to those obtained in the double gel-diffusion. Absorbed anti-BLH serum reacted with NIH-LH-B5 and Fraction 5 of the gel filtration of BLH, unabsorbed antiserum reacted with NIH-LH-B5 and Fraction 1 of the gel filtration of BLH (Figure 2).

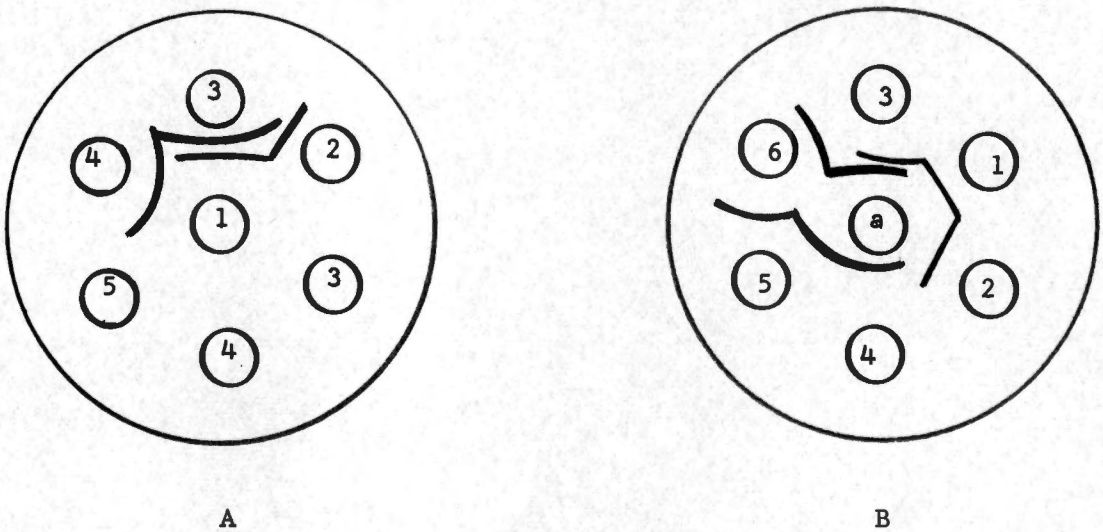

Figure 3. Double gel-diffusion precipitin test showing relationship between absorbed, unabsorbed anti-BLH sera and BLH, purified BLH fractions. A: Well 1 NIH-LH-B5 30 µg; Well 2 bovine serum; Well 3 anti-BLH serum 30 λ; Well 4 absorbed anti-BLH serum 30 λ; B: Well a anti-BLH serum 30 λ; Well 3 NIH-LH-B5 30 µg; Well 1, 2, 4, 5 NIH-LH-B5 fractions 1, 2, 4, 5 from Sephadex G-100 column (Figure 2, page 13); Well 6 absorbed anti-BLH serum.

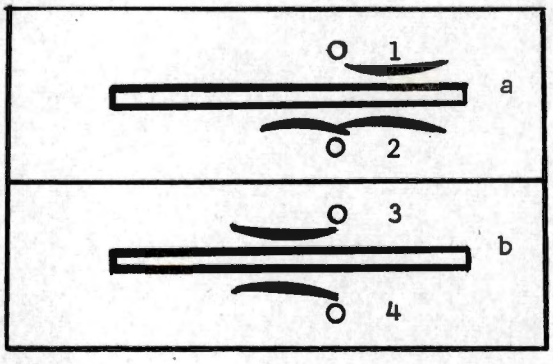

Figure 4. Immunoelectrophoresis of bovine LH. Trough a unabsorbed anti-BLH serum; Trough b absorbed anti-BLH serum; Well 1, 4 NIH-LH-B5 fractions 1, 5 from Sephadex G-100 column (Figure 2, page 13); Well 2, 3 NIH-LH-B5 4 µg.

Preparation of ^{131}I-labeled BLH. Purified BLH (Fraction 5 from Sephadex G-100 column, Figure 2, page 13) was dissolved in 0.05 M phosphate buffer pH 7.5, at a concentration of 60 µg. per ml. and stored at -25°C. in 0.2 ml. aliquots for labeling with ^{131}I. Three µg. of the BLH solution (0.05 ml.) was iodinated with 2 to 3 mc. of ^{131}I (New England Nuclear Corp., Boston, Mass.) by the method described by Greenwood, Hunter and Glover (1963) using 100 µg. of Chloramine-T as oxidizing agent. Separation of ^{131}I-labeled BLH from free iodide was accomplished using a Sephadex G-50 column of 1.0 cm. x 13 cm. The column was previously equilibrated with 0.07 M barbitone buffer, pH 8.6 and saturated with 20 mg. of crystallized-lyophilized ovalbumin in 1 ml. barbitone buffer. The ovalbumin was followed by an additional 20 ml. of buffer. The separation was conducted at 4°C. with fraction size of 5 drops which was equivalent to 0.4 ml. per minute. The result is shown in Figure 5; specific activities of iodinated hormone was estimated to be from 140 to 200 µc. per µg. Fractions from the front edge of the first peak were used in the assays and the LH-^{131}I was always utilized within 24 hours of preparation.

Radioimmunoassay procedures. The radioimmunoassay technique for human LH described by Wilde, Orr and Bagshawe (1967) and Odell, Ross and Rayford (1967b) was modified. The nature of reactions between BLH antibodies and ^{131}I-labeled BLH, between BLH antibody and unlabeled BLH, and between BLH-bound antibody and second antibody are depicted in Figure 6. The protocol of procedures for the radioimmunoassay as used

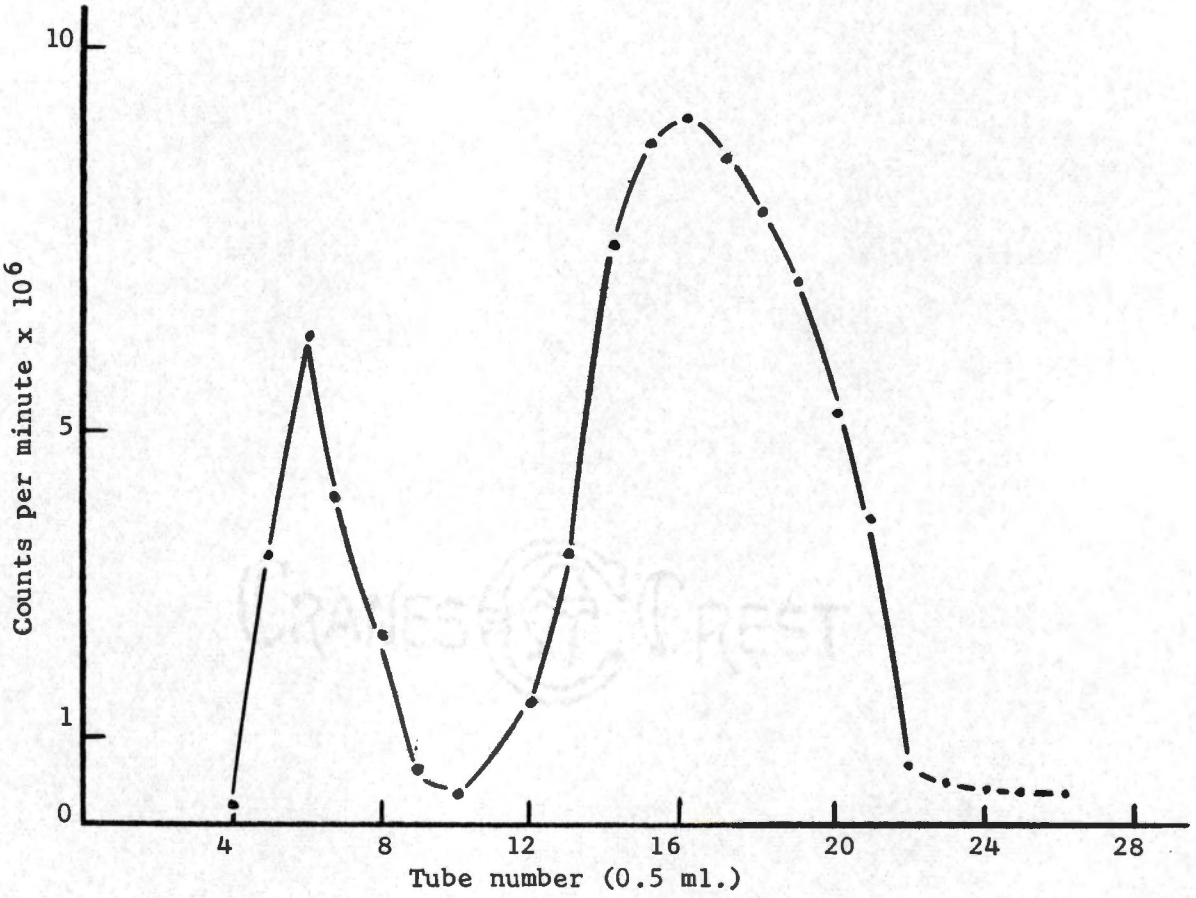

Figure 5. Separation of ^{131}I-labeled BLH from ^{131}I-iodide on Sephadex G-50. Separation was carried out by gel filtration of the iodination reaction mixture from a 1.0 cm. x 13.0 cm. column of Sephadex G-50. The column was presaturated with 20 mg. in 1.0 ml. of crystallized and lyophilized ovalbumin buffer, pH 8.6. Each point represented the radioactivity contained in each consecutive 0.5 ml. elute expressed as counts per minute (CPM). Flow rate was 0.4 ml. per minute.

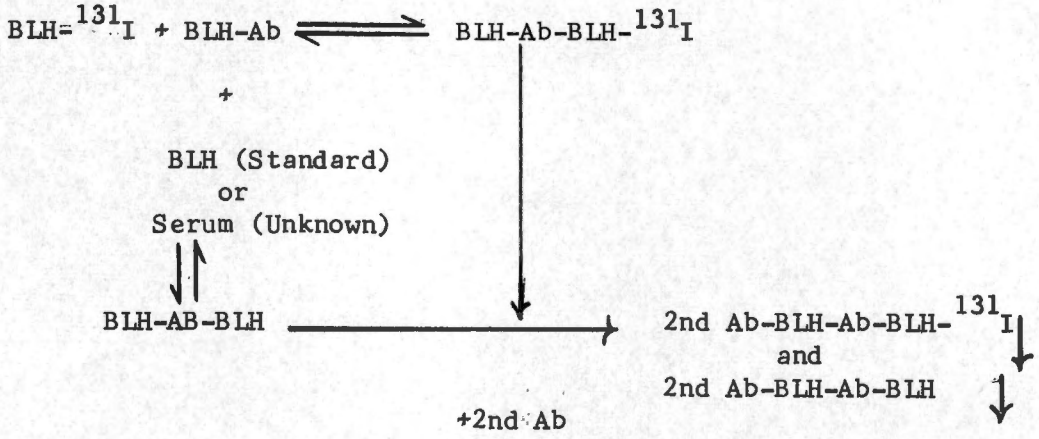

Figure 6. Diagram showing competition between unlabeled standard BLH or unknown serum and labeled BLH-^{131}I for binding anti-BLH serum. Addition of 2nd Ab (sheep anti-rabbit γ-globulin antibody) shows visible precipitation.

in this study are shown in Figure 7. Incubations were carried out in 15 mm. x 110 mm. glass tubes (Nuclear-Chicago, Des Plaines, Illinois), and all reagents were added to the tubes in the following order: (1) 20λ , 100λ or 200λ of each serum sample to be assayed (i.e., 40λ to 400λ of standard hormone preparations), (2) 100λ of 0.1 M ethylenediaminetetracetic acid (EDTA), ph 7.6, (3) 100λ containing 0.1 to 0.3 mμg. of ^{131}I-BLH, (4) 100λ of anti-BLH serum diluted to 1:8000 (final dilution 1:80000), (5) diluent to make a total volume of 1.0 ml. and (6) this mixture was incubated at 4°C. for 5 days. On the fifth day, 100λ of sheep anti-rabbit-γ-globulin serum with a dilution of 1:2 was added to each tube, the mixture was incubated further for 24 hours at 4°C., reaction tubes were then-centrifuged at 500 g. for 20 minutes and the supernatant was removed by suction. The radioactivity in the precipitate was measured in an automatic gamma spectrometer (Model 1085, Nuclear-Chicago, Des Plaines, Illinois).

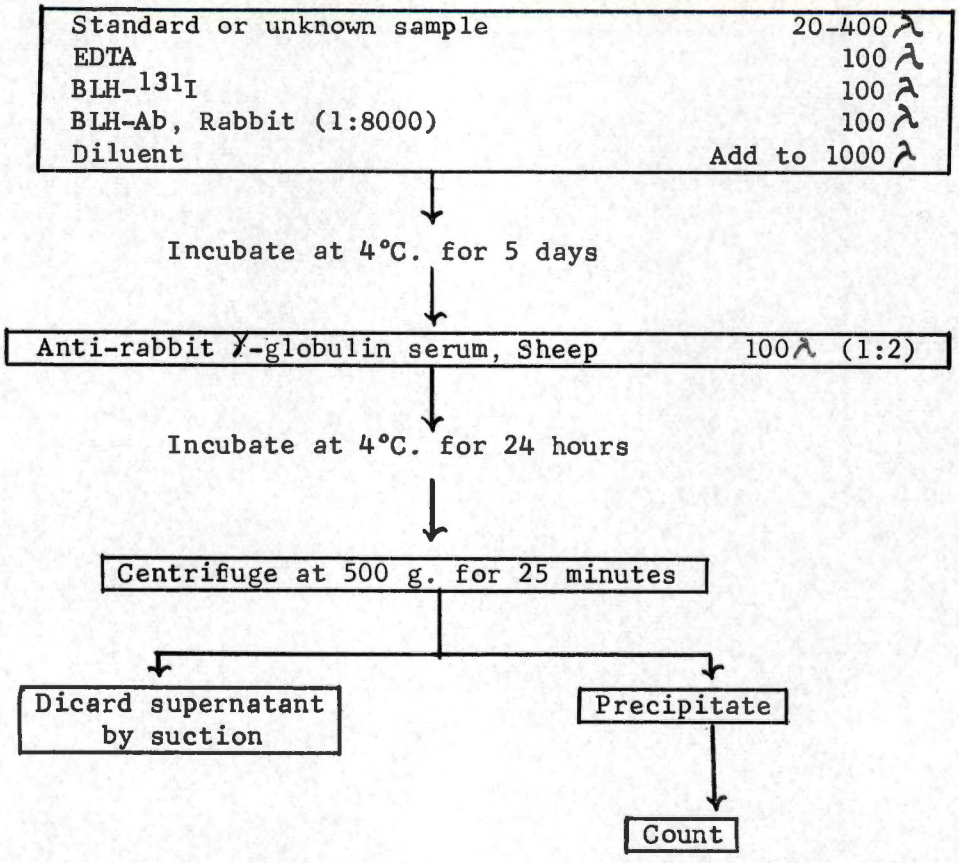

Figure 7. Double antibody system for BLH immunoassay. Flow chart showing the steps of radioimmunoassay for bovine luteinizing hormone (BLH).

CHAPTER IV

RESULTS AND DISCUSSION

Titration of rabbit anti-BLH serum. The potency of rabbit anti-BLH serum was estimated by incubating 700 λ of diluent, 100 λ of EDTA, 100 λ of labeled BLH with 100 λ of anti-BLH serum in dilution of 1:1000 to 1:16000 for 5 days at 4°C. On the fifth day, 100 λ of the second antibody (sheep anti-rabbit γ-globulin serum) diluted 1:2 was added to the above mixture and incubated an additional 24 hours. The radioactivity recovered from the precipitate decreased gradually from a 1:1000 dilution to a 1:8000 dilution of anti-BLH serum; a relatively sharp reduction of radioactivity in the precipitate was observed at the dilution between 1:8000 and 1:16000 (Figure 8). The highest dilution of anti-BLH serum having the most efficiency for recovering the radioactivity in the precipitate was estimated at 1:8000; consequently, this dilution of anti-BLH serum was used for assaying standard preparations as well as unknown serum samples.

Incubation periods. The effect of the length of incubation period on the recovery of ^{131}I-labeled BLH in the precipitate was determined by mixing a constant amount of ^{131}I-labeled BLH and anti-BLH serum (diluted 1:8000), EDTA solution and an adequate amount of diluent to make a total reaction mixture of 1 ml. These samples were divided into two groups, A and B. The duration of the incubation period for both groups

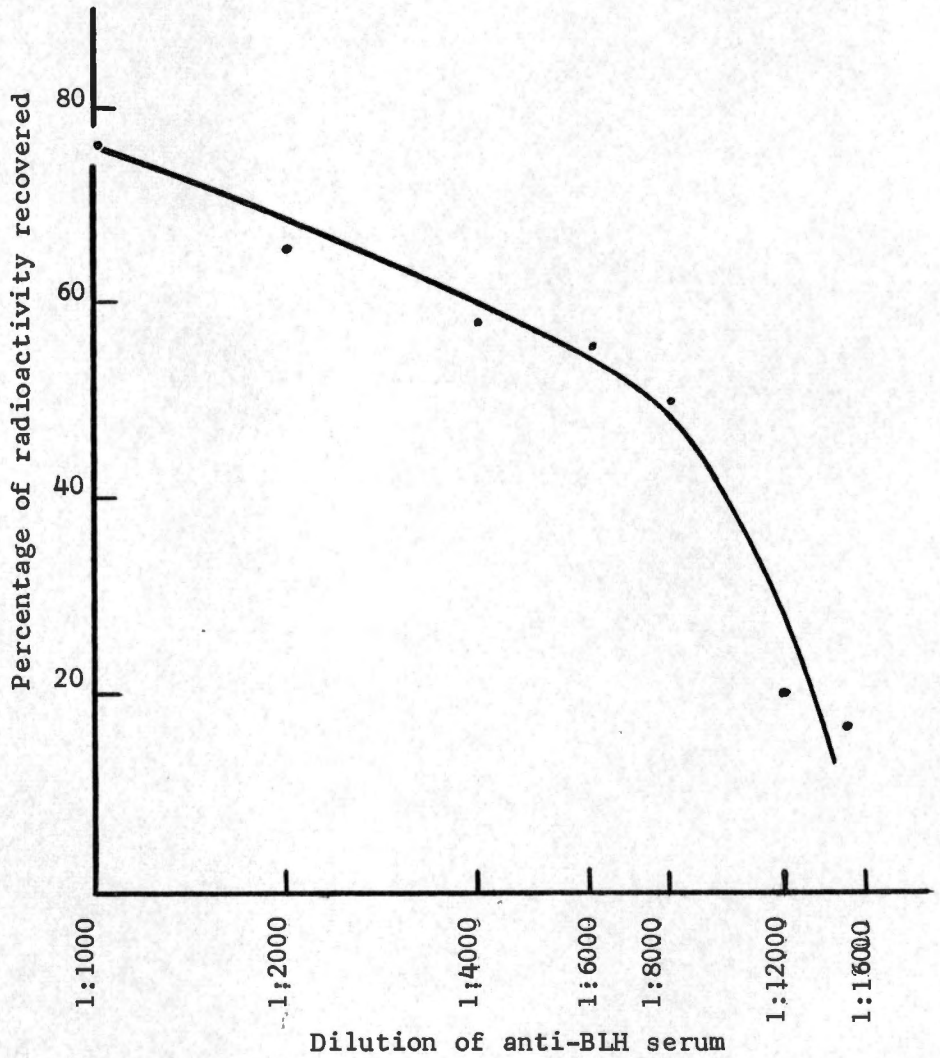

Figure 8. Titration of rabbit anti-BLH serum. The effect of the concentration of rabbit anti-BLH serum on the recovery of labeled BLH in the precipitate, expressed as a percentage of the total radioactivity added. Second antibody added at a dilution of 1:2.

varied from 0 to 6 days. Group A was incubated for 24 hours and Group B for 48 hours after the addition of 100λ of a second antibody and the radioactivity of the precipitate was measured. Incubation was at 4°C. The maximum radioactivity was recovered from Group A after 5 days incubation and from Group B after 4 days incubation (Figure 9). Total incubation time for both groups was 6 days. An additional 24-hour incubation (Group B) did not increase the recovery of radioactivity in the precipitate. This result suggests that the reaction betweeh BLH and its antibody is continuous and the reaction between BLH-bound antibody and second antibody is instantaneous. This result agrees with the findings of Wilde, Orr and Bagshawe (1967).

According to the result obtained from this experiment, the following procedure was adopted for assaying standard preparations and unknown samples. The reaction mixture of ^{131}I-labeled BLH, anti-BLH serum, EDTA and diluent was incubated at 4°C. for 5 days. On the fifth day, a second antibody was added and incubation was continued for 24 hours.

Standard dose-response curve. Serial 1:10 dilutions of unlabeled NIH-LH-B5 were prepared. Aliquots ranging in volume from 40λ to 400λ which contained from 0.005 to 150 mμg. of the hormone were mixed with a constant amount of ^{131}I-labeled BLH, EDTA, anti-BLH serum and diluent to make a total volume of 1.0 ml. The mixture was incubated at 4°c. for 5 days. On the fifth day of incubation, a second antibody was added and incubation was continued for 24 hours.

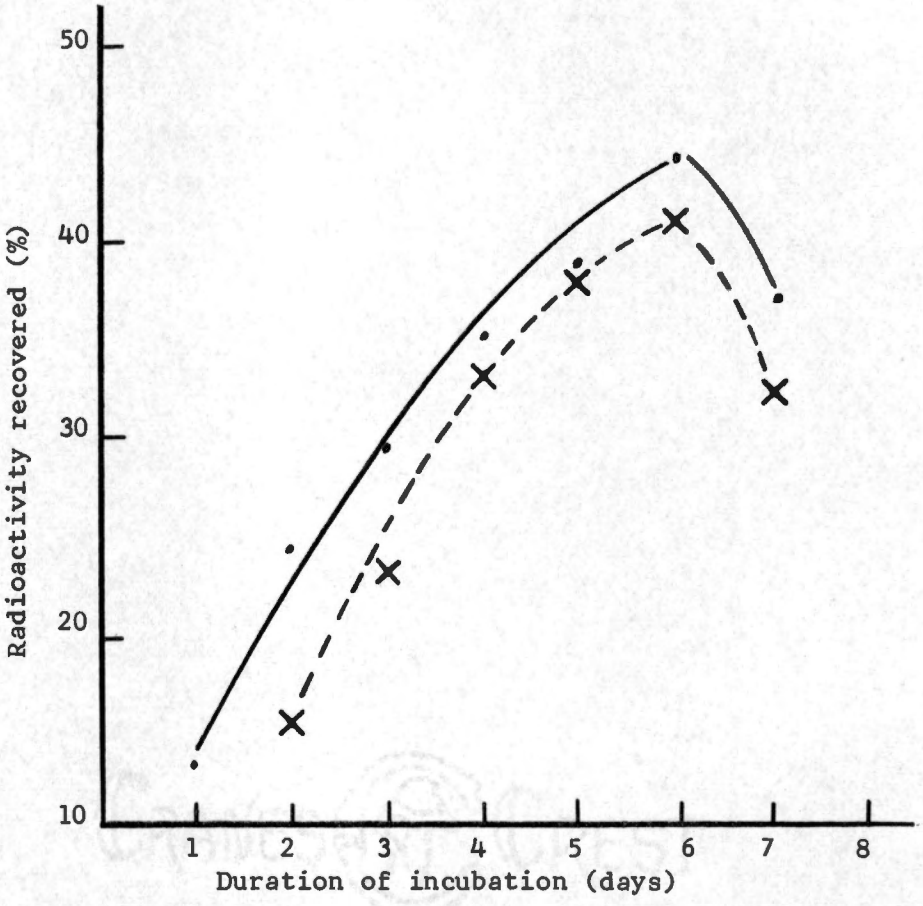

Figure 9. Duration of incubation. Effect of the duration of incubation on the recovery of ^{131}I labeled BLH in the precipitate is epxressed as a percentage of the total radioactivity added. Anti-BLH serum at a dilution of 1:8000: second antibody at 1:2 dilution; ● counted 24 hours after the addition of second antibody; x counted 48 hours after the addition of second antibody.

A typical standard dose-response curve is shown in Figure 10. The background count in the blank tubes containing all components of the reaction mixture, except anti-BLH serum, was defined as 0 percent. There was no ^{131}I-labeled BLH bound to antibody in the blank tubes. All background counts were subtracted from counts in all standard references as well as unknown samples. The number of counts in the precipitate in tubes containing only ^{131}I-labeled BLH and its antibody but no unlabeled hormone was defined as 100 percent.

A steep portion of curve was obtained over a range of 0.05 to 100 mμg. of standard NIH-LH-B5 preparation, which represented 85 to 15 percent of radioactivity in the precipitate as compared to that in the tubes containing only ^{131}I-labeled BLH and its antibody. Only values between 85 and 15 percent were used for the estimation of LH in serum samples.

LH level in bovine serum. Twelve to fourteen blood samples were collected from each of the four cows. Cows were checked with a vasectomized bull twice a day, usually at 8:00 a.m. and 3:00 p.m. The onset of estrus was designated as day 1; blood was collected at the first detection of estrous behavior and at 12-hour intervals during estrus. All four cows were first found in estrus in the morning and through the afternoon but out of estrus the next morning. A total of seven estrous periods were identified; three cows came back into estrus within 24 days, one cow (720) did not return to estrus within 26 days and no further collections of blood were made. The morning sample taken at the first estrus of cow 114 was lost. The afternoon samples were obtained at

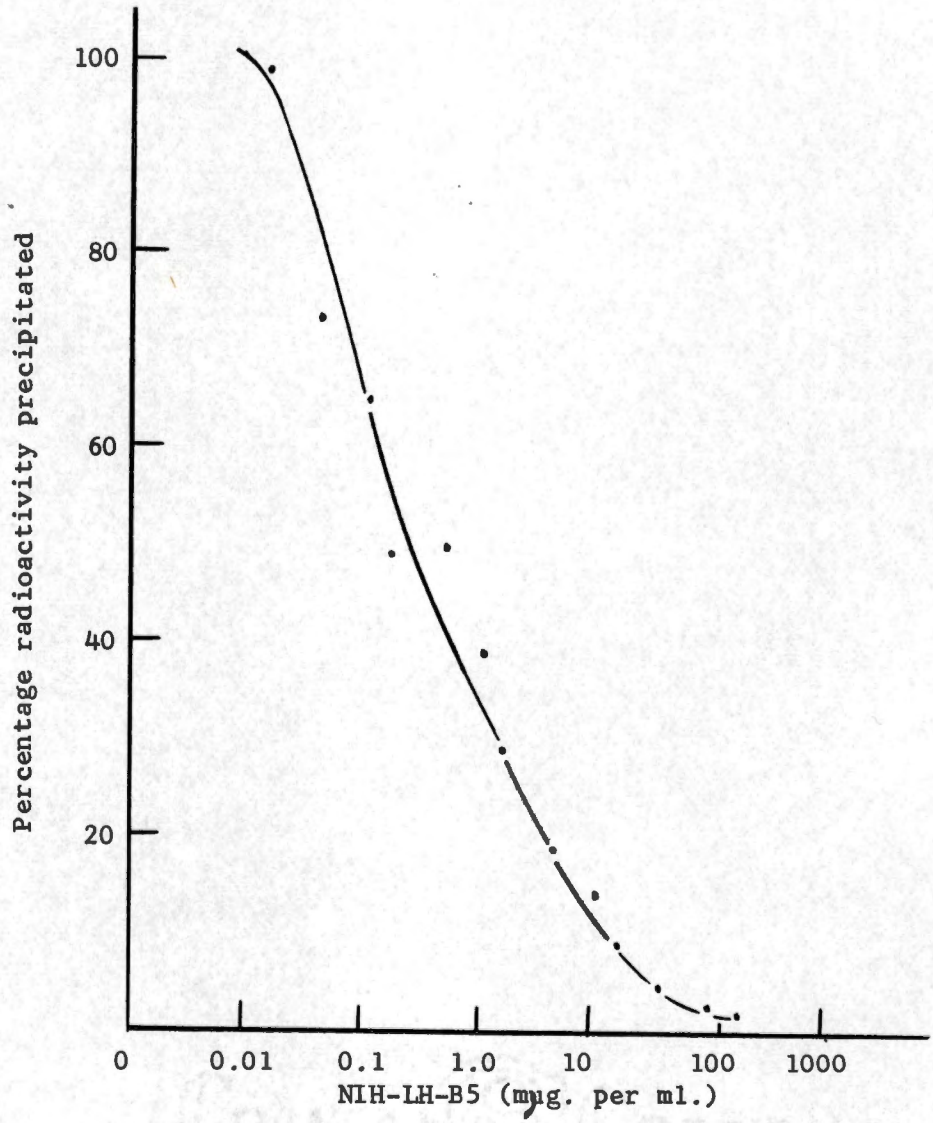

Figure 10. Standard curve on semilogarithmic plot obtained by addition of known amounts of unlabeled NIH-LH-B5 to a fixed amount of antibody and LH-^{131}I. Separation of antibody bound BLH from free BLH-^{131}I was accomplished by a double antibody technique. The number of counts precipitated in tubes containing only LH-^{131}I and antibody was defined as 100 percent.

about 3:30 p.m. Cow 1308 came back into estrus on Saturday and the only sample was collected at 11:00 a.m. Cow 1490 returned to estrus on Saturday and the blood was collected at 1:00 p.m. only.

The double antibody technique was employed to measure the concentration of LH in 53 serum samples from the four cows. With the exception of one sample from cow 1490 on the 16th day, all samples were assayed at least two separate times. In the first assay 20, 100 and 200λ aliquots of serum were used and in subsequent assays 20 and 180λ aliquots of serum were used. The LH concentrations in the blood through the cycle of four cows is shown in Table I. The averages of 0.70, 1.52, 1.07 and 0.98 mμg. per ml. of serum were obtained during diestrus from cows 114, 720, 1490 and 1308, respectively; these values increased by 10 to 20 fold during the estrous period.

The changes in the LH level of these cows are shown individually in Figures 11 through 14. The peak LH levels in the sera were found in the early stages of estrus and dropped sharply in the later stages except for cow 1308 in which the LH peak occurred near the end of estrus. The trends in LH levels were quite similar in all seven estrous cycles. Cow 720 did not show any significant elevation of LH within the subsequent 26 days after the initial estrous period.

The length of the estrous cycle in the cow is about 21 days and the duration of estrus is 13 to 17 hours; ovulation occurs 12 to 15 hours after the end of estrus (Nalbandov, 1964). In this study the LH was elevated during estrus and returned to the diestrus level within

TABLE I

LUTEINIZING HORMONE IN BLOOD SERUM OF COWS DURING THE ESTROUS CYCLE

Day of cycle	**LH mug. per ml. serum, mean ± SE			
	Animal number			
	114	1490	720	1308
1†	*5.35 ± 1.07	*6.25 ± 1.65	*21.7 ± 5.00	*1.92 ± 1.29
1†		*1.43 ± 0.70	* 3.27 ± 2.62	*8.30 ± 0.75
2	0.48 ± 0.07	0.90 ± 0.54	3.6 ± 2.88	15.40 ± 4.65
3	0.87 ± 0.14	0.54 ± 0.02		
4				0.64 ± 0.12
5	0.55 ± 0.02	1.11 ± 0.37	0.86 ± 0.40	
6				0.83 ± 0.33
7		0.88 ± 0.14	0.89 ± 0.22	
9	0.55 ± 0.14	0.82 ± 0.12	0.72 ± 0.02	1.09 ± 0.43
11				0.99 ± 0.42
12	0.47 ± 0.18	0.70 ± 0.24	1.58 ± 0.83	
13				0.74 ± 0.27
14	0.67 ± 0.23	2.85 ± 0.68	1.34 ± 0.69	
16	0.92 ± 0.02	0.4	2.18 ± 1.52	0.84 ± 0.38
18		*4.4 ± 1.26		1.70 ± 0.96
19	0.73 ± 0.24	0.5 ± 0.14	1.96 ± 0.86	
20				0.34 ± 0.06
21	0.53 ± 0.20		1.24 ± 0.34	*6.00 ± 2.00
22				1.16 ± 0.42
23	0.51 ± 0.24		1.97 ± 0.94	0.42 ± 0.16
25†	*12.73 ± 3.85			
25†	* 8.37 ± 3.85			
26	1.46 ± 0.56		2.40 ± 1.32	

† = Samples were collected twice daily.

** = NIH-LH-B5 equivalents.

* = At estrus

Number of determinations ranged from 1 to 4 per blood sample with an average of 3.01.

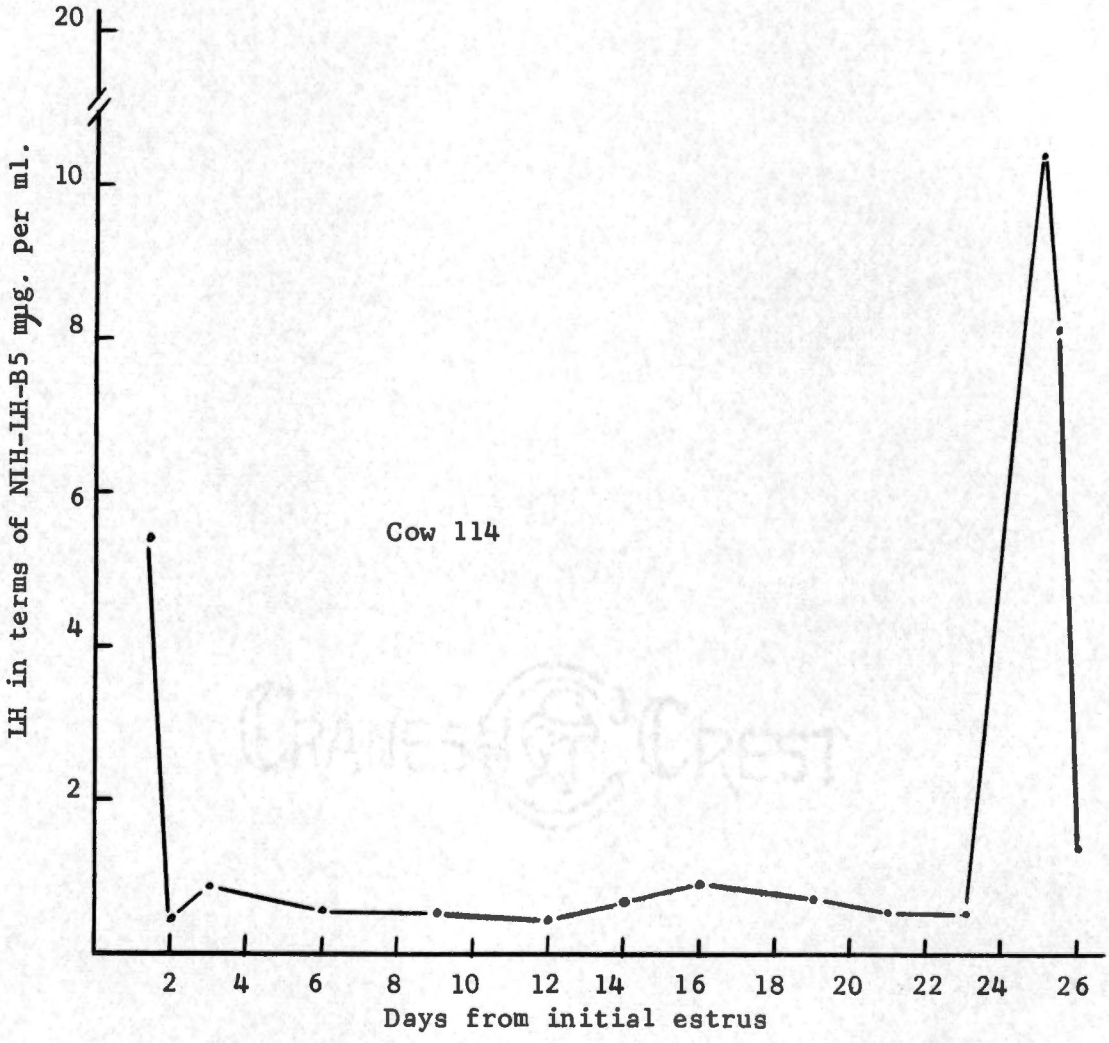

Figure 11. Bovine serum LH from cow 114 (Holstein) during estrous cycle as measured by radioimmunoassay. Sample collections were initiated during one estrous period and continued periodically until 1 day after the next estrous period.

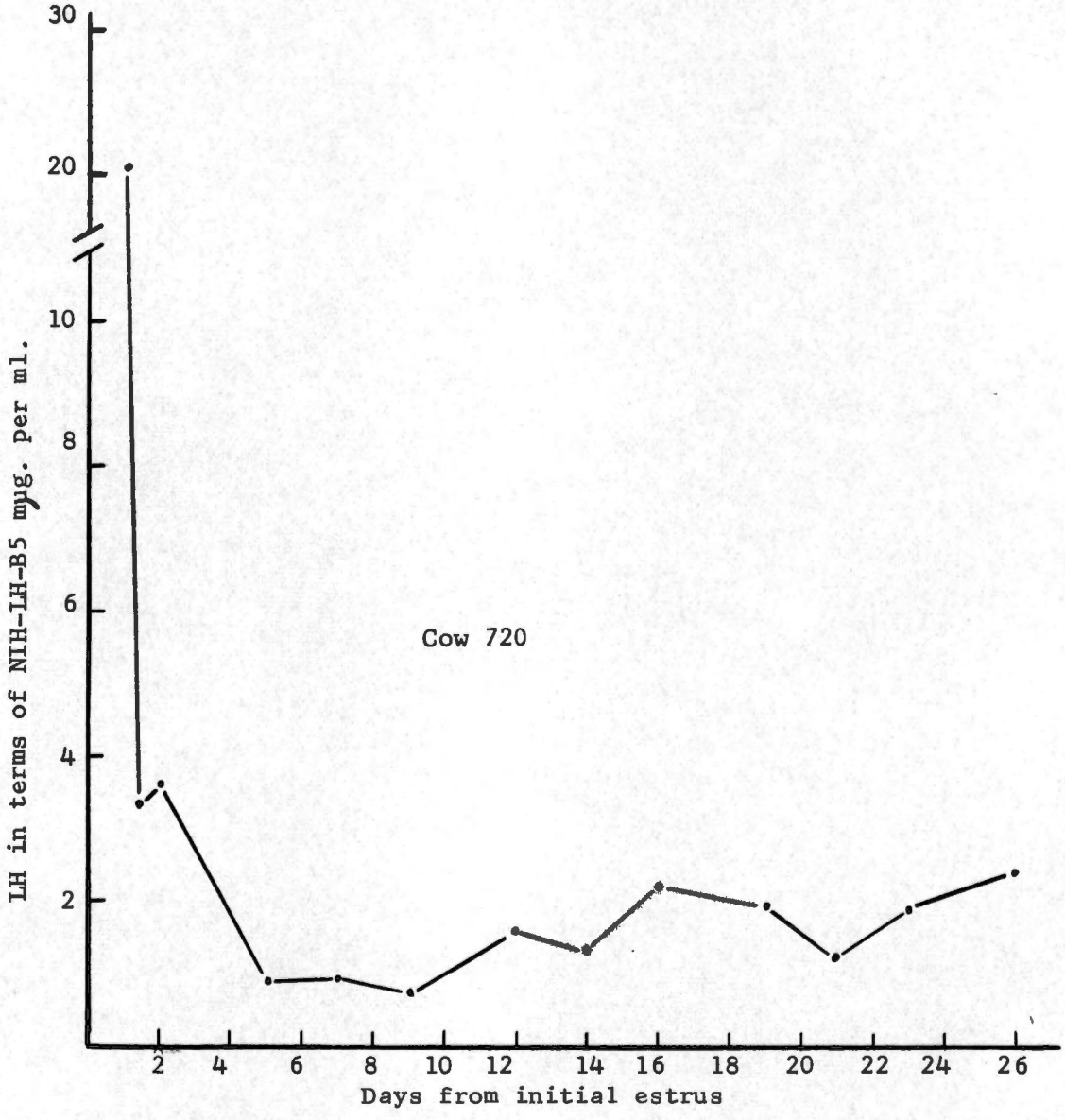

Figure 12. Bovine serum LH from cow 720 (Hereford) during estrous cycle as measured by radioimmunoassay. Sample collections were initiated during one estrous period and continued periodically until 1 day after the next estrous period.

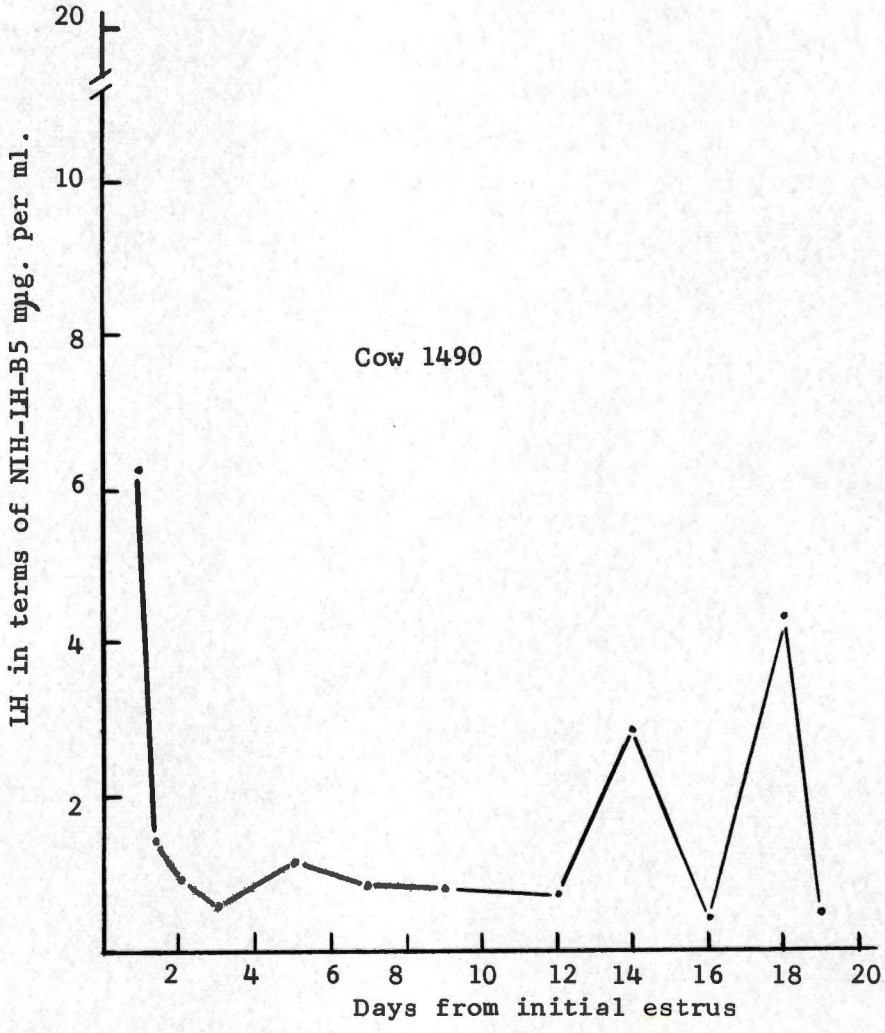

Figure 13. Bovine serum LH from cow 1490 (Hereford) during estrous cycle as measured by radioimmunoassay. Sample collections were initiated during one estrous period and continued periodically until 1 day after the next period.

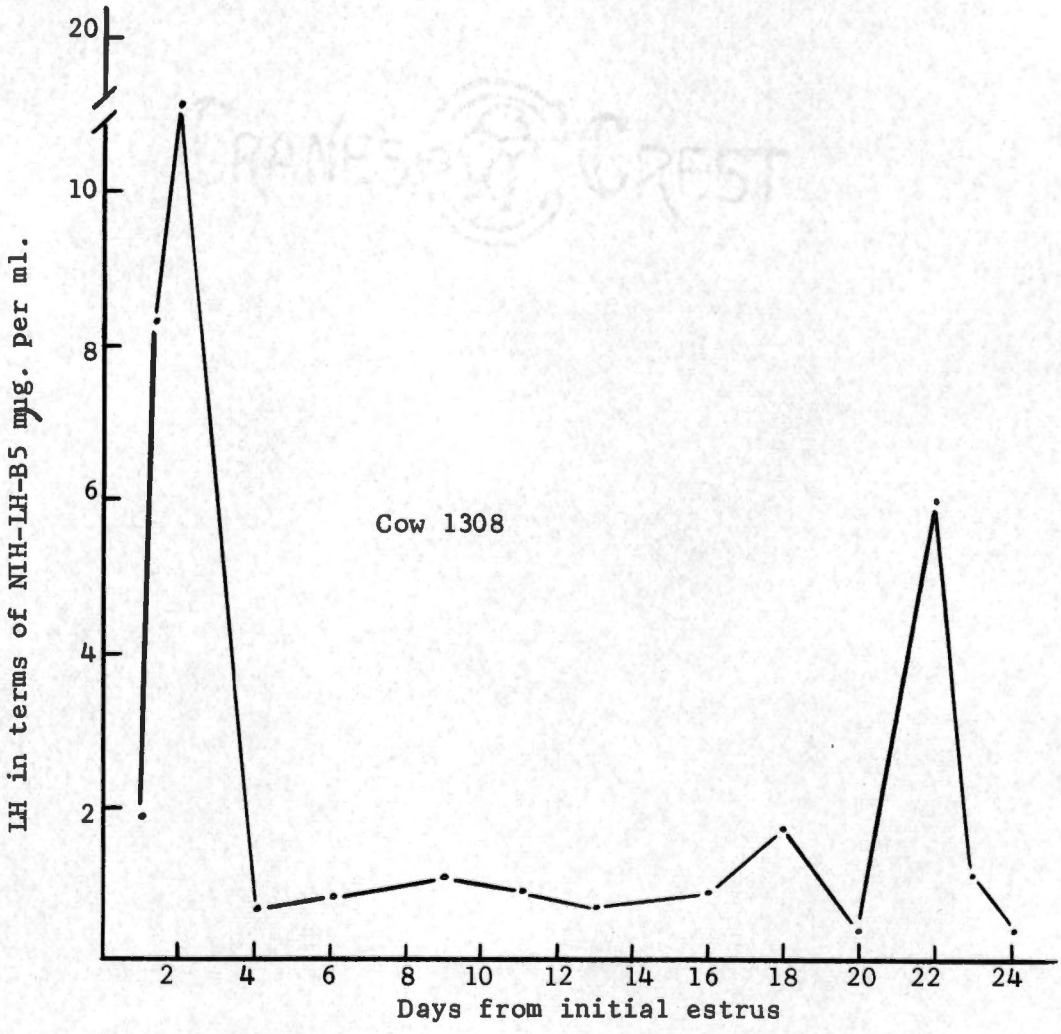

Figure 14. Bovine serum LH from cow 1308 (Angus-Hereford cross) during estrous cycle as measured by radioimmunoassay. Sample collections were initiated during one estrous period and continued periodically until 1 day after the next period.

24 hours. These data suggest that the LH returned to the diestrus level prior to or by the time of ovulation and agree with the report of Varian, Henricks and Melampy (1967). The time during which LH was elevated in the vlood was estimated to be about 6 to 12 hours. This agrees with the finding of Anderson and McShan (1966) that there was almost no LH in the blood obtained 21 hours after the first sign of estrus. In order to establish the detailed changes in blood levels of LH during the estrous period, blood samples should be collected at frequent intervals prior to the onset of estrus and through at least one day after the end of estrus.

The concentration of LH in the blood stream is low and transitory. Only two reports of attempts to estimate blood levels were found (Anderson and McShan, 1966; Varian, Henricks and Melampy, 1967). Both groups used the OAAD method which required concentration of the LH prior to assay. Anderson and McShan indicated that a minimum of 100 ml. of whole blood was required for a single test. Recently, the specificity of the OAAD procedure for LH has been questioned. Bell, Mukerji and Loraine (1962) indicated it gave reliable results with highly purified preparations derived from pituitary tissue and urine but was less accurate when relatively crude urinary extracts were tested. Anderson (1965 reported that under some experimental conditions a humoral agent, not LH, can cause ovarian ascorbic depletion in the test animals. Although the OAAD procedure is, currently, the most widely used assay for LH in pituitary tissue and human pregnancy urine, the necessity for concentration from blood and the question of specificity for LH raises a major question as to the applicability of

this technique for assay of LH in blood.

Does the "standard LH," extracted from pituitary tissue by chemical procedures, and the blood LH, not subjected to chemical manipulations react in the same manner and to the same degree with antibodies? There is evidence of differences between the immunological and biological activity of protein hormones. Neuraminic acid is essential for the biological activity of the follicle-stimulating hormone. If this hormone is treated with neuraminidase it loses almost all biological activity but is still immunologically active; also LH, which has been biologically inactivated by periodate, remains immunologically reactive (Wilde and Bagshawe, 1965). The labeled antigen has to be biologically active and the antibody has to be capable of reacting with the biologically active hormone in the radioimmunoassay system. If biologically inactive antigens, which may be metabolic products or may result from the chemical extraction, are present the bioassay-immunoassay ratio will depend entirely on the ratio of concentration of such antigens in the standard and unknown samples. The parallelism of the radioimmunoassay and bioassay needs to be validated. However, due to (1) the lack, at the present time, of chemically pure preparations of LH to be used both as the standard for the bioassay and as the antigen for immunoassay; (2) the very low levels of LH in the blood necessitating extraction and concentration prior to bioassay and (3) the inherent errors in any bioassay procedure, such a study would be difficult to evaluate.

The specificity of the radioimmunoassay for human LH has been evaluated by Odell, Ross and Rayford (1967a). Dose-response curves similar to the curve for human LH was obtained when purified human TSH or FSH was used instead of LH. However, 20 and 100 times as much TSH or FSH, respectively, as LH were required to give the same response. This phenomenon was attributed to contamination of the TSH and FSH preparations with small amounts of LH. A similar result was reported by Parlow et al., (1965) employing the OAAD test to evaluate the purity of LH preparations. Extracts of human pituitary glands, containing LH, FSH and TSH were purified by CM-C, Sephadex G-100 and DEAE column chromatography. Even though the final preparation of LH was three times as potent as the NIH standard (NIH-LH-S1) it still contained small amounts of TSH and FSH. Even though it appears to be almost impossible to chemically separate all FSH and TSH from LH preparations, the very low levels of these contaminants does not appear to invalidate the use of this assay system for LH in the blood.

Cross-reactivity among various species has been reported. Rabbit antiserum to ovine LH has been shown to give a positive precipitin reaction in agar-gel with the homologous LH as well as with bovine, rat and pig pituitary LH but not with human LH (Moudgal and Li, 1961). Neutralization of endogenous LH in the normal rabbit with rabbit antiserum to ovine LH blocked ovulation (Quadri, Harbers and Spies, 1966). Rabbit antiserum to bovine pituitary LH neutralized the biological activity of the homologous hormone as well as the LH from human, ovine, porcine and equine species,

but the positive immunological reactions were observed only with bovine, ovine and porcine LH (Reichert and Treadwell, 1967). Odell, Ross and Rayford (1966) measured the LH in human plasma employing radioimmunoassay by taking advantage of the cross-reaction between human LH and HCG. The importance of the degree of cross-reactivity will account for the feasibility of the application of measuring hormones in one species by utilization of hormones from other species; however, further characterization of the species relationships in a quantitative study will be required in order to resolve this point.

The effect of iodination on the hormone has been studied. When Utiger, Parker and Daughaday (1962) used 30 to 80 mc. of ^{131}I to label insulin and nitrous acid as the oxidizing agent, they observed denaturation and degradation of the hormone. This was assumed to be due to the oxidizing agent and the high amount of ^{131}I used. The electrophoretic pattern of labeled growth hormone on starch-block electrophoresis showed a slight broadening of the peak and a slight increase in mobility towards the anode (Greenwood and Hunter, 1963). When they used Chloramine-T as the oxidizing agent and 4 to 6 mc. or 2 mc. of ^{131}I to label 5 µg. of growth hormone they obtained specific activities of 533 to 748 mc. per mg. as compared to 200 to 300 mc. per mg., respectively. The former preparation exhibited a decreased affinity for the antiserum, whereas the latter preparation was indistinguishable from the unlabeled hormone.

Based on the finding of Greenwood and Hunter (1963) and the quantity of ^{131}I (2 mc. per 3 µg. of hormone) used in the current study,

it was assumed that the reactivity of the ^{131}I labeled BLH was not affected.

Although ^{131}I has been used for the majority of studies as a tracer for labeling antigens, ^{125}I has also been used as a tracer in the radioimmunoassay system. ^{125}I has not yet achieved acceptance as the choice for isotopic labeling probably because of lower specific activity. ^{125}I with its longer physical half-life and lower gamma energy emission has led to its employment for isotopic labeling. Hormone preparations labeled with ^{125}I are as useful in radioimmunoassay for human LH as their ^{131}I counterparts containing several fold greater specific activities (Toshihiro et al., 1967). Presumably, the same would be true for other protein compounds.

The results obtained in the current study show that this procedure can be used to measure the level of LH in the blood of the cow. The small quantity of blood required makes possible serial analyses of frequent intervals. Harris, Hilary and Bagshawe (1967) indicated that they have assayed up to 30 samples per hour. Based on the experience gained in the current study it is estimated that a technician can assay as many as 150 samples per day. If refinements can be made, i.e., automation in dispensing and mixing of reagents, the numbers of samples assayed can be further increased.

Application of this technique to the study of other protein hormones offers an excellent opportunity to better understand the complex interrelationships involved in the growth and reproduction of animals.

CHAPTER V

SUMMARY

A radioimmunoassay for the quantitative determination of bovine luteinizing hormone is described. The assay is based on the competition between ^{131}I-labeled BLH and unlabeled BLH for anti-BLH antibodies. The separation of free hormone from anti-BLH serum-bound hormone is achieved by precipitation using anti-rabbit γ-globulin serum of sheep. The assay was used to measure variations of BLH in serum of four cows throughout the estrous cycle. The average levels of LH were 0.70, 0.98, 1.07 and 1.51 mμg. equivalents of NIH-LH-B5 per ml. of serum during diestrus, but increased by 10 to 20 fold during estrus in all four cows. The results obtained from blood samples collected at 12-hour intervals at estrus and at 2 to 3 days intervals during diestrus suggest that the concentration of LH in the blood increases sharply in the early stages of estrus and drops rapidly in the late stage of estrus. The time during which LH remains high in the blood was estimated to be about 6 to 12 hours. These results indicate that the procedure can be used to measure the LH activity in 1 ml. of blood without concentration and purification.

LITERATURE CITED

LITERATURE CITED

Anderson. R. C. 1965. Studies of the reliability and specificity of the ovarian ascorbic acid depletion assay for luteinizing hormone. Ph. D. Thesis, Yale University, Dissertation Abstracts 20:6811.

Anderson. R. R. and W. H. McShan. 1966. Luteinizing hormone levels in pig, cow and rat blood plasma during the estrous cycle. 78:976.

Bagshawe, K. D., G. E. Wilde and A. H. Orr. 1966. Radioimmunoassay for human chorionic gonadotropin and lutenizing hormone. Lancet 1:1118.

Bell, E. T., S. Mukerji and J. A. Loraine. 1964. A new bioassay method for luteinizing hormone depending on the depletion of rat ovarian cholestrol. J. Endocr. 28:321.

Berson, S. A., R. S. Yalow, A. Bruman, M. A. Rothschild and K. Newerly. 1956. Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin-treated subjects. J. Clin. Invest. 35:170.

Berson, S. A., R. S. Yalow, G. D. Aurbach and J. T. Potts, Jr. 1963. Immunoassay of bovine and human parathyroid hormone. Proc. Nat. Acad. Sci. 49:613.

Berson, S. A., and R. S. Yalow. 1964. Immunoassay of protein hormones. In: The Hormones (G. Pincus, K. V. Thimann and E. B. Astwood, eds.). Academic Press. Vol. V. Chapter XI. p. 557, New York.

Boyden, S. V. 1951. Adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exp. Med. 93:107.

Campbell, D. H., J. S. Garvey, N. E. Cremer and D. H. Sussdorf. 1964. Methods in immunology. W. A. Benjamin, Inc., New York.

Collip, J. B. and E. M. Anderson. 1934. Production of serum inhibitory to thyrotropic hormone. Lancet 1:76.

Collip, J. B. 1935. Recent studies on anti-hormones. Ann. Internal. Med. 9:150.

Felber, J. P. 1963. ACTH antibodies and their use for radioimmunoassay for ACTH. Experientia. 19:227.

Glick, S. N., J. Roth, R. S. Yalow and S. A. Berson. 1963. Immunoassay of human growth hormone in plasma. Nature 199:784.

Greenwood, F. C., W. M. Hunter and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. II. 89:114.

Harris, B. F., A. Hilary and K. A. Bagshawe. 1967. Automated radioimmunoassay for HCG and LH. Technican Symposium, New York. p. 4.

Hayashida, T. and C. H. Li. 1958a. Immunological investigations on bovine pituitary growth hormone. Endocrinology 63:487.

Hayashida, T. and C. H. Li. 1958b. An immunological investigation of human pituitary growth hormone. Science 128:1276.

Hunter, W. M. and F. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature Lond. 194:495.

Leathem, J. H. 1949. The antihormone problem in endocrine therapy. In Recent Prog. Hormone Res. 4:115.

Midgley, A. R., Jr. and R. B. Jaffe. 1966. Human luteinizing hormone in serum during the menstrual cycle: Determination by radioimmunoassay. J. Clin. Endocr. 26:1375.

Midgley, A. R. 1967. Radioimmunoassay for human follicle-stimulating hormone. J. Clin. Endocr. 27:295.

Morgan, C. R. and A. Lazarow. 1962. Immunoassay of insulin using a two-antibody system. Proc. Exper. Biol. Med. 110:29.

Moudgal, N. R. and C. H. Li. 1961. An immunochemical study of sheep pituitary interstitial cell-stimulating hormone. Arch. Biochem. Biophys. 95:93.

Nalvandov, A. V. 1964. Reproductive physiology. 2nd Ed. W. H. Freeman and Co., San Francisco.

Odell, W. D., J. F. Wilber and W. E. Paul. 1965. Radioimmunoassay of thyrotropin in human serum. J. Clin. Endocr. 25:1179.

Odell, W. D., G. T. Ross and P. L. Rayford. 1966. Radioimmunoassay for human luteinizing hormone. Metabolism. 15:287.

Odell, W. D, G. T. Ross and P. L. Rayford. 1967a. Radioimmunoassay for luteinizing hormone in human plasma or serum: Physiological studies. J. Clin. Invest. 46:248.

Odell, W. D., G. T. Ross and P. L. Rayford. 1967b. Simplified partially automated method for radioimmunoassay for human thyroid-stimulating. growth, luteinizing, and follicle stimulating hormones. J. Lab. and Clin. Med. 70:973.

Parlow, A. F. 1961. Bioassay of pituitary inteinizing hormone by depletion of ovarian ascorbic acid. In Human Pituitary Gonadotropins (A. Albert, Editor). Charles C. Thomas, Springfield, Illinois, p. 300.

Parlow, A. F., P. G. Condliffe, L. E. Reichert, Jr. and A. E. Wilhelmi. 1965. Recovery and partial purification of FSH and LH during the purification of TSH from human pituitary glanda. Endocr. 76:27.

Paul, W. E. and W. D. Odell. 1964. Radiation inactiviation of the immunological and biological activities of human chorionic gonadotropin. Nature, Lond. 203:979.

Quadri, S. K., L. H. Harbers and H. G. Spies. 1966. Inhibition of spermatogenesis and ovulation in rabbits with antiovine LH rabbit serum. Soc. Exp. Biol. Med. 123:809.

Read, C. H. and G. T. Bryan. 1960. The immunological assay of human growth hormone. Recent Progr. Hormone Res. 16:187.

Reichert, L. E., Jr. and P. E. Treadwell. 1967. Cross-reactivity of antibody to human and bovine luteinizing hormone (LH) with homologous and heterologous LH. Endocr. 81:591.

Skom, J. H. and D. W. Talmage. 1958. Non-precipitating insulin antibodies. J. Clin. Invest. 37:783.

Tashihiro, A., D. P. Goldstein, M. L. Taymor and K. Dolch. 1967. A radioimmunoassay method for human pituitary luteinizing hormone (LH) and human chorionic gonadotropic (HCG) using ^{125}I-labeled LH. Amer. J. Obstet. Gynec. 98:996.

Thompson, K. W. 1941. The antihormones. Physiol. Rev. 21:588.

Utiger, R. D., M. R. Parker and W. H. Daughaday. 1962. Studies of human growth hormone. I. A radioimmunoassay for human growth hormone. J. Clin. Endocr. 41:254.

Utiger, R. D. 1964. The radioimmunoassay of plasma thyrotropin in hyperthyroidism and hypothyroidism. J. Lab. Clin. Med. 64:1013.

Varian, N. B., D. M. Henricks and R. M. Melampy. 1967. OAAD activity (LH) in bovine plasma. J. Ani. Sci. 26:1496. Abstr.

Wilde, C. E., A. H. Orr and K. D. Bagshawe. 1965. A radioimmunoassay for human chorionic gonadotropin. Nature, Lond. 205:191.

Wilde, C. E., A. H. Orr and K. D. Bagshawe. 1967. A sensitive radioimmunoassay for human chorionic gonadotrophin and luteinizing hormone. J. Endocr. 37:23.

Yalow, R. S. and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39:1157.

Yalow, R. S., S. M. Glick, J. Roth and S. A. Berson. 1964. Radioimmunoassay of the human plasma ACTH. J. Clin. Endocr. 24:1219.

VITA

Pin-Kwen Chung was born in Taiwan, China in 1933. After completion of high school in southern Taiwan, he entered National Taiwan University, pursuing a B. S. in Veterinary Science and Animal Husbandry. After graduation in 1958, he went to the armed service for one and a half years as a second lieutenant in the Quartermaster Corps. Upon leaving the service, he was employed as research assistant at the dairy farm of National Taiwan University for three years.

He came to the United States in 1963 and entered The University of Tennessee, received a M. S. degree in 1965 from the Department of Animal Husbandry and Veterinary Science and completed his Ph. D. program at The University of Tennessee-Atomic Energy Commission Agricultural Research Laboratory. Presently, he is associated with Pentex Incorporated at Kankakee, Illinois.