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DEVELOPMENT OF A RADIOIMMUNOASSAY TO MEASURE LUTEINIZING HORMONE IN BOVINE BLOOD

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OPSOMMING: ONTWIKKELING VAN 'N RADIO-IMMUNOLOGIESE BEPALINGSMETODE VIR LUTEÏNISERENDE HORMOON IN BLOED VAN DIE BEES

'n Radio-immunologiese bepalingsmetode vir luteïniserende hormoon (LH) in bloed van die bees is ontwikkel duer die gebruik van buisies bestryk met teëliggame. Antisera teen bees-LH (NIH-LH-B7) is in konyne verwek. Nie-spesifisiteit is uit die gekombineerde antisera verwyder deur adsorpsie. 'n Beskrywing word gegee van die betroubaarheid van die bepalingsmetode in fisiologiese studies.

SUMMARY:

A radioimmunoassay using tubes coated with antibody was developed to measure luteinizing hormone (LH) in bovine blood. Antisera to bovine LH (NIH-LH-B7) were raised in rabbits. Non-specificity of pooled antisera was removed from the antisera by adsorption. The reliability of the assay for use in physiological studies is described.

The mammalian oestrous cycle is regulated mainly by the endocrine system which operates through an integrated series of feedback mechanisms. Consequently, knowledge of functional levels of relevant hormones is essential to understand the various physiological changes which occur during the cycle. Information about blood levels of gonadotrophin hormones is of particular value in studies on the physiological control of oestrus and ovulation and on the causes of dysfunction associated with these phenomena. The gonadotrophin complex comprises follicle stimulating hormone (FSH) and luteinizing hormone (LH). Prolactin may or may not be considered to be an integral part of this complex. At present, these hormones can be measured conveniently in peripheral blood of cows only by radioimmunoassay.

Radioimmunoassay of bovine LH requires:

1. Highly purified bovine LH for radiolabelling;
2. Antiserum to bovine LH which has adequate affinity, avidity and specificity for bovine LH;
3. Radiolabelled LH which is prepared by reaction of highly purified bovine LH with a radioisotope. The immunological properties of the radiolabelled LH molecule must not be altered by this reaction;
4. A standard preparation of LH;
5. A method to separate antibody-bound from unbound radiolabelled LH in the radioimmunoassay reaction mixture.

This paper describes how these requirements were met during development of a radioimmunoassay to measure levels of LH in peripheral blood of cows through the oestrous cycle.

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Procedure

1. *Antisera*

Purified bovine LH (LER1072-2) was used for all radio-iodinations. FSH (NIH-FSH-S9), thyrotrophin (TSH) (NIH-TSH-B3) and LH (NIH-LH-B7) were used for standards and NIH-LH-B7 as an antigen to raise antisera against bovine LH.

(a) *Production of antisera*

Antisera to bovine LH were raised in 5 rabbits. Initially, 500 µg NIH-LH-B7 in 1,0 ml saline and emulsified with 1,0 ml Freund's complete adjuvant (Difco Laboratories, Detroit) was injected subcutaneously in the region of the inguinal (0,4 ml each) and popliteal (0,6 ml each) lymph nodes. Booster injections, which contained 1 mg NIH-LH-B7 in 1,0 ml saline and 1 ml Freund's complete adjuvant, were administered similarly 10, 20, 60 and 70 days after the initial injection. Blood was collected from the lateral vein of the ear before each booster injection and 10 days after the final injection. Sera were collected and stored (-10^{o}) until required.

(b) *Tests for antibodies to LH and for specificity of antisera*

Sera were tested for presence of antibodies to LH by immunodiffusion. Ionagar No. 2 (Oxoid, London) (1,0%) was dissolved in 0,05 M barbital buffer pH 8,6 by heating and 3,2 ml of this solution was placed on a microscope slide (25 mm x 77 mm) and allowed to gel. Wells were cut in the gel. Cross-reactivity of antisera to LH was studied by examination of formation of precipitin bands in the gel between wells containing sera and wells containing various antigens. These antigens were aqueous extracts of bovine kidney, liver, heart, spleen and brain and bovine serum albumen, male calf serum, ovine FSH (1 mg/ml), bovine TSH (1 mg/ml) and bovine LH in various concentrations.

Table 1

Production of antisera and the effect of a second booster injection of NIH-LH-B7 on titre of anti LH antisera in the double antibody radio-immunoassay

a) Sera collected before 2nd booster injection

Antiserum dilution		% added ^{125}I - LH bound by antisera						Titre
		1:1 000	1:5 000	1:10 000	1:40 000	1:80 000	1:160 000	
Antiserum no.	1194	43,5	17,3	11,9	7,4	5,4	4,7	< 1 000
	1195	11,5	6,3	8,2	7,4	5,9	3,5	< 1 000
	1196	44,7	20,1	14,6	9,8	6,9	8,4	< 1 000
	1197	51,9	27,1	18,5	8,9	5,8	7,0	± 1 000
	1198	16,9	6,9	6,0	4,6	2,9	4,2	< 1 000

b) Sera collected 10 days after 2nd booster injection

Antiserum dilution		% added ^{125}I – LH bound by antisera						Titre
		1:1 000	1:5 000	1:10 000	1:40 000	1:80 000	1:160 000	
Antiserum no.	1194	75,0	49,1	42,9	24,9	16,2	9,8	5 872
	1195	34,6	22,6	16,9	18,3	13,2	9,1	< 1 000
	1196	96,1	65,8	60,3	42,6	30,7	19,5	20 398
	1197	99,5	78,4	71,1	38,2	24,5	14,5	21 986
	1198	68,2	50,6	43,6	20,6	21,3	12,0	4 983

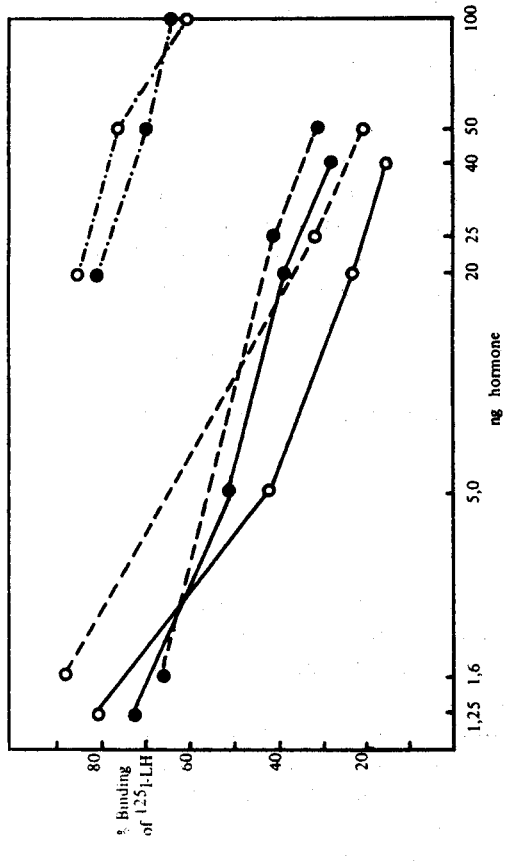

Fig. 1 *The effect of presence of TSH, FSH and LH on the proportion (%) of labelled LH bound by unadsorbed antisera in a double antibody radioimmunoassay*
● ● Antiserum 1196; o o Antiserum 1197
—— LH; – – – – TSH; —·— FSH.

Precipitin bands were formed between all wells containing sera and wells containing LH. Precipitin bands were also formed by immunodiffusion against male calf serum, tissue extracts and bovine TSH. Limited cross-reaction was noted with high concentrations of ovine FSH.

Since immunodiffusion provides only qualitative information about immunological cross-reactivity of antisera, all sera from the first and second bleedings were tested by radioimmunoassay to determine titre. Antisera were diluted 1:1 000, 1:5 000, 1:10 000, 1:40 000, 1:80 000 and 1:160 000 in 0,05 M phosphate buffer, pH 7,5. An aliquot of each dilution (0,2 ml) was incubated with radiolabelled bovine LH as described by Niswender, Reichert, Midgley & Nalbandov (1969). Titre was taken as that dilution of antiserum which bound 50% of labelled LH added to assay tubes which contained no unlabelled LH (assay blank tubes) (Table 1). Titre of antisera increased greatly after the second booster injection.

Cross-reactivity with bovine TSH and ovine FSH was determined by the double antibody radioimmunoassay of Niswender *et al.* (1969). Antisera were used at a dilution which bound 50% of added labelled LH in assay blank tubes. Standard curves were determined by inclusion of standard amounts of ovine FSH, bovine TSH and bovine LH (Fig. 1). All antisera showed a high degree of cross-reactivity with TSH.

The two antisera with highest titre against bovine LH (1196 and 1197) were pooled and adsorbed with various extracts to remove this cross-reactivity as described below:

(c) *Adsorbtion of antisera*

Calf serum (0,25 ml/ml antiserum) was added to the pooled antiserum and incubated at room temperature for 2,5 h. with occasional shaking. Acetone-dried bovine liver powder (Sigma, St. Louis) (200 mg powder/ml antisera) was added to the mixture which was incubated for a further 4 h. at room temperature, again with occasional shaking. The mixture was placed in a refrigerator (4°), incubated overnight, then centrifuged at 15 000 g for 10 min (4°) in a 30S rotor. The supernatant was collected. When tested by immunodiffusion, the adsorbed antiserum formed precipitin bands only against bovine TSH and LH.

The pooled adsorbed antiserum was further adsorbed with NIH-TSH-B3 (20 mg TSH/ml serum) for 2 h. at room temperature and overnight at 4°. Antiserum was centrifuged as before. Immunodiffusion against TSH resulted in a slight precipitin band. Consequently, adsorbtion with TSH was repeated. The resultant antiserum was used in all further procedures.

2. *Radiolabelling of LH*

In initial studies, the method of Niswender *et al.* (1969) was used to label 2,5 µg LH (LER 1072–2) with ^{125}I. This method used only 20 µg chloramine T. Although successful at first, this method led to unsatisfactory and inconsistent binding of ^{125}I to hormone in later studies. The method of Greenwood, Hunter & Glover (1963) was found to give more reliable results when modified as follows. LH (2,5 µg LER 1072–2) in 25 µl 0,05 M phosphate buffer pH 7,5 (PB) was incubated with 1 mci ^{125}I (IMS 30, Radiochemical Centre, Amersham) in the presence of 50 µg chloramine T in 20 µl PB for 40 sec. Reaction was stopped by addition of 120 µg sodium metabisulphite in 50 µl PB to the reaction mixture. Potassium iodide (150 µg in 75 µl PB) was added. The reaction mixture was transferred to a column (300 x 10 mm) of Sephadex G–75 which had been previously equilibrated with PB, saturated with 1 ml 5% bovine serum albumen (BSA) in PB and washed thoroughly with PB (at least 3 bed volumes). The reaction mixture was eluted from the column with PB. Fractions of 1 ml were collected into tubes containing 0,1 ml 5% BSA in PB in 0,15 M NaCl (5% BSA-PBS). Aliquots (50 µl) of each fraction were counted in a gamma spectrometer (1 sec) and the location of the "protein" (elution volume 10–20 ml) and "free iodine" (elution volume 28–38 ml) peaks determined by measurement of radioactivity in the fractions. The "protein" fraction was pooled and diluted with 0,1% BSA-PBS until 0,1 ml diluted labelled LH provided ± 30 000 counts per minute.

3. *Separation of antibody-bound from unbound radiolabelled LH*

The most commonly used methods to separate antibody-bound and free labelled hormone in radioimmunoassays are:

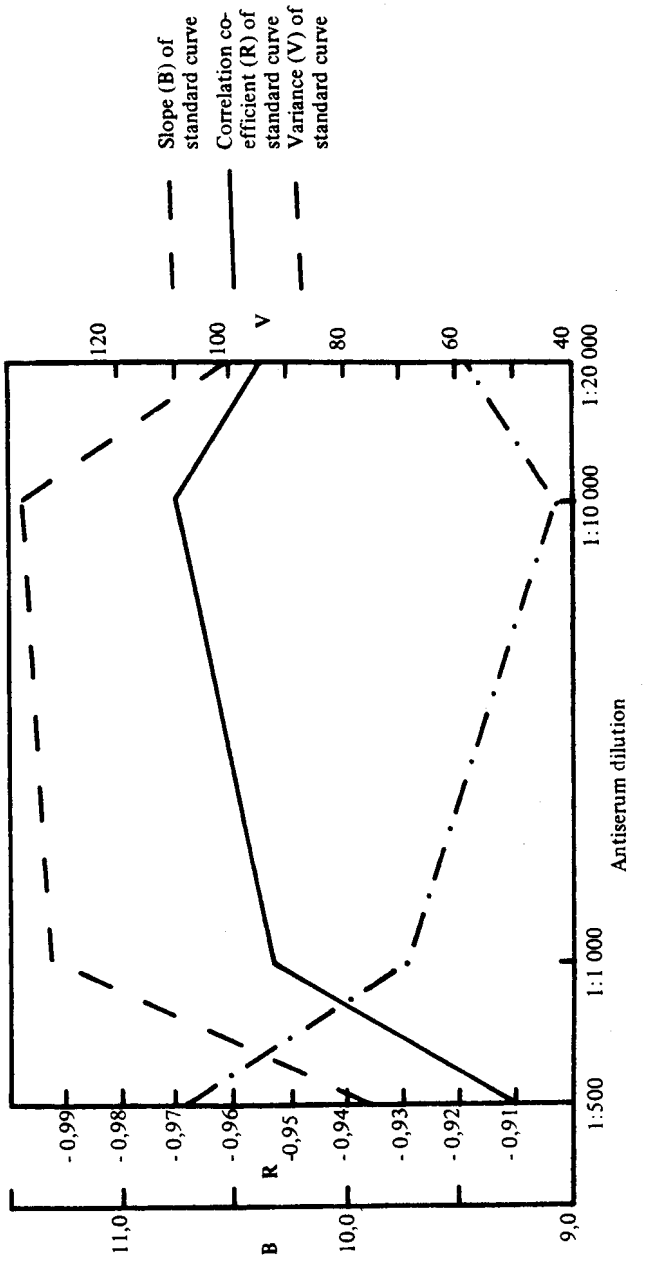

Fig. 2 *The effect of dilution of antiserum on the slope, correlation coefficient and variance of the standard curve of the coated tube radioimmunoassay for bovine LH*

1. Double antibody method.
2. Dextran-coated charcoal method.
3. Coated tube method.

In preliminary studies, the double antibody method of Niswender *et al.* (1969) was used in development of a radio-immunoassay for bovine LH. Antiserum was used at a dilution of 1:80 000. This dilution of antiserum bound 42,3% of labelled LH added to assay blank tubes. At a dilution of 1:160 000, 29,3% labelled LH was bound.

Although the double antibody method gave accurate and repeatable results, the method required a total of 5 days for completion. This comprised incuba-

tion for 3 d of antiserum with standards or serum samples followed by incubation for 2 d after addition of a second antibody (goat anti-rabbit-gammaglobulin). By contrast, solid-phase radioimmunoassays require only 24 h or less. Thus, solid-phase radioimmunoassays are more convenient for processing the large numbers of samples which are involved in study of the bovine oestrous cycle.

Use of dextran-coated charcoal to separate antibody-bound from free labelled hormone was studied. Antiserum (0,2 ml) diluted 1:80 000 was added to each assay tube. Labelled LH (0,1 ml) was added with 0,5 ml 0,25% BSA-PBS. Addition of 1 ml dextran-coated charcoal suspension (containing 25 mg charcoal and 2,5 mg dextran in 0,25% BSA-PBS) and subsequent centrifugation at 1 600 g led to adsorbtion of all the labelled LH. Thus, this method was not suitable for use with the antiserum produced in this laboratory. However, this technique provided satisfactory dose response curves when an equine anti-bovine-LH antiserum (donated by Dr. Snook) was used. Using this antiserum, minimum detectable quantity of LH was 0,6 ng LH when the antiserum was diluted 1:10 000. However, addition of protein (BSA) to the assay system resulted in spuriously high results. For these reasons the dextran-coated charcoal method was abandoned and a coated tube method (Catt & Tregear, 1967) was adopted for radioimmunoassay of bovine LH.

Three types of polystyrene tube which were available locally were found to be suitable for use in the coated tube procedure – Thrombo test tubes (Luckham, Brussels), E2 tubes (Stayne Laboratories, Buckinghamshire) and 73/TT ROR 3 ml tubes (Unoplast, Copenhagen). Use of our antiserum diluted 1:10 000 in 0,05 M bicarbonate buffer pH 9,6 to coat tubes (1 ml diluted antiserum/tube) resulted in binding of 30–40% of added labelled LH. At this dilution, the standard curve had greater slope and correlation coefficient and lower variance than at greater or lesser dilutions of antiserum (Fig. 2).

4. *Assay procedure*

1. Antiserum (1 ml), diluted 1:10 000 in 0,05 M bicarbonate buffer pH 9,6 was added to each plastic assay tube. Tubes were incubated for 16 h (or overnight) at room temperature. Bicarbonate buffer (1 ml) without antiserum was added to other tubes for determination of non-specific binding.
2. Tubes were washed three times with 1,4 ml 0,15 M NaCl and 0,5% BSA-PBS (1,2 ml) was added to the tubes which were kept refrigerated (4°) for at least 24 h before use.
3. When required for assay, tubes were aspirated.
4. Standard curve. Standard NIH-LH-B7 was diluted with 0,1% BSA-PBS so that requisite amounts (25, 12,5, 6,25, 3,12, 1,56, 0,78 or 0,39 ng) were contained in 1 ml diluent which was added to each standard tube. Assay blank tubes contained 1 ml 0,1 % BSA-PBS. A standard curve with duplicate tubes was included for each 200 tubes in the assay.
5. Samples. Sample volume varied with LH concentration of the sample. For daily bovine serum samples, 0,5 ml serum was added to each assay tube. For hourly blood samples taken during oestrus (Hale 1974a), 0,2 ml plasma was added to the assay tube. For assay of samples of high concentration of LH (e.g. samples from *in vitro* incubation of pituitary tissue) sample volume could be as small as 10 µl. Volume of the sample was made up to 1,0 ml with 0,1% BSA-PBS diluent. Each sample was assayed in duplicate.
6. LH (LER 1072–2) labelled with ^{125}I (±30 000 counts/min/0,1 ml) in 0,1 ml 0,1% BSA was added to all tubes.
7. Tubes were vortex-mixed for 10 sec and incubated for 16 h at room temperature.
8. Tubes were emptied by inversion over a sink using a wire mesh to retain tubes in their racks which each held 500 tubes.
9. Tubes were washed 6 times with running tap water and were emptied by inversion after each washing. Tubes were allowed to drain until dry by inversion on blotting paper.
10. Radioactivity remaining attached to the tubes (labelled hormone-antibody complex) was determined by counting tubes in an automatic gamma spectrometer. Standard curves were determined and hormone content of samples with 95% confidence limits estimated using a programmable electronic calculator.

Results

1. *Deterioration of labelled LH*

The proportion of labelled LH which was bound by antibody in assay blank tubes decreased with increase in time after labelling (Fig. 3A). Percentage binding of labelled LH decreased by half within one week of labelling. Thereafter, percentage binding remained relatively constant for a further week. Similarly, slope of the standard curve declined during the first week after labelling (Fig. 3B). As a result, precision of the assay was greatest when freshly labelled hormone was used and declined thereafter as the labelled hormone preparation deteriorated. The correlation coefficient of the standard curve was affected little by decline in slope of the curve. Correlation coefficients were always greater than −0,96. Since one technician can handle 1 000 determinations each day, radiolabelling was routinely carried out on Mondays and the labelled hormone preparation was used in approximately 5 000 determinations during the next five days. Adequate numbers of tubes were coated during the week before labelling.

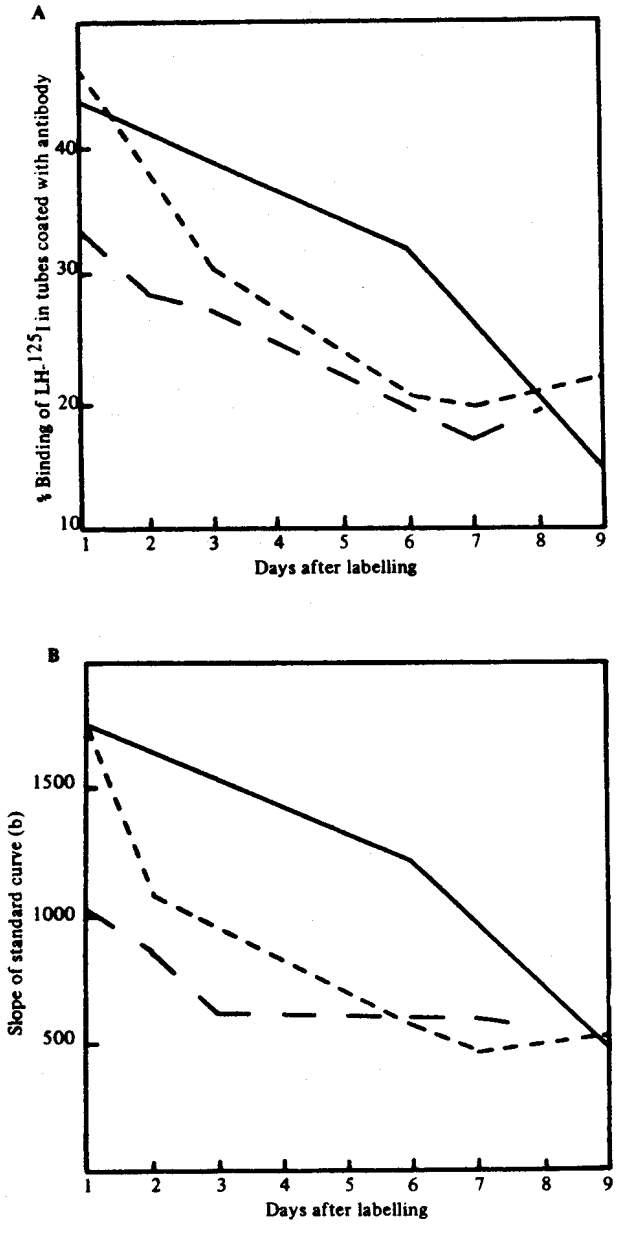

Fig. 3 *The effect of age of labelled LH on A) proportion (%) of labelled hormone bound by plastic tubes coated with adsorbed anti LH antiserum diluted 1:10 000 and B) the slope (b) of the standard curve. Each line represents one preparation of labelled bovine LH*

2. *Reliability of the assay*

The percentage of added labelled LH which was bound by antibody was plotted graphically against log of the amount of unlabelled LH in assay tubes. The resultant curve was parallel with the curve derived similarly from serial dilution of a bovine blood sample (Fig. 4). Furthermore, after correction for amount of LH in the serum, the standard curve was not affected significantly by presence of 0,5 ml bovine serum in the assay

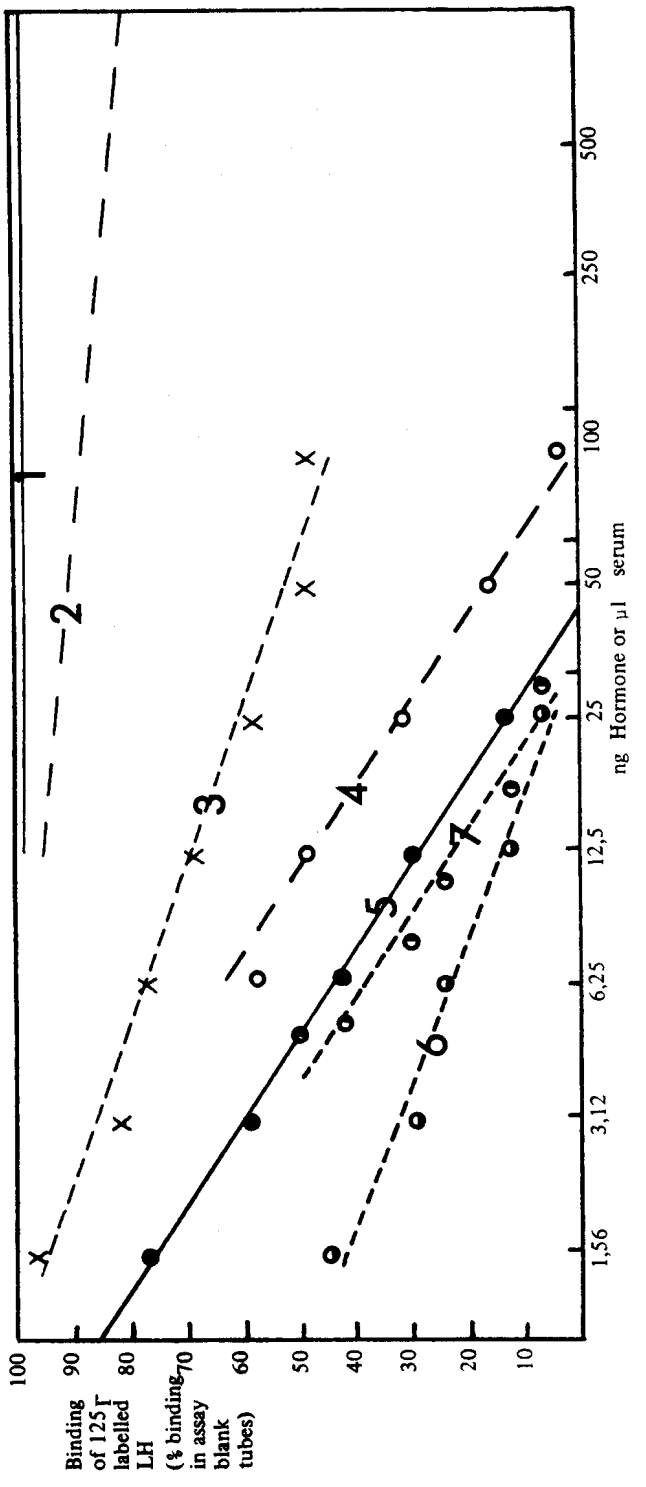

Fig. 4 *The effect of presence of various antigens on the binding of 125 I-labelled LH by assay tubes coated with anti LH antiserum*

1. ——— *Prolactin*
2. – – – – – *NIH-FSH-S9*
3. x– – –x *NIH-TSH-B3*
4. o– – –o *Bovine serum containing the equivalent of 54,4 ng NIH-LH-B7/ml.*
5. ●——● *NIH-LH-B7*
6. ●– – –● *NIH-LH-B7 in the presence of 0,5 ml bovine serum containing the equivalent of 9,0 ng NIH-LH-B7/ml.*
7. ●– – –● *Response line number 6 after correction for LH content of bovine serum*

tube. The adsorbed anti bovine LH antiserum cross-reacted with bovine TSH. However, the slope of the response curve to TSH was less steep than that of the response curve to LH ($P < 0,01$). As a result, estimates of affinity of the antiserum for TSH varied between 5 % and 19% of its affinity for LH, depending upon dose of TSH. Cross-reactions with bovine prolactin and ovine FSH were low. Thus, the assay is adequately specific for measurement of LH in bovine blood samples and pituitary extracts and incubation media. After log transformation of dose, the standard curve of the assay was linear between 0,4 and 50 ng LH/assay tube.

Sensitivity of the assay varied with age of the labelled LH preparation. Using freshly labelled LH, the assay was adequately sensitive and precise to distinguish 200–400 pg NIH–LH–B7 as being significantly different from 0 pg LH.

Coefficient of variation of estimates varied with individual assay between 5 and 20%. Confidence limits ($P < 0,05$) of determinations represented a range of approximately one increment in the standard curve (e.g. for an estimate of 7,2 ng LH/ml serum derived from duplicate samples, 95% confidence limits were 5,2 to 10,0 ng LH/ml serum).

Discussion

Ability to measure functional levels of LH in bovines is of unquestionable importance. Since no commercial radioimmunoassay kits are available to allow this to be done, it was necessary to develop a radioimmunoassay in this laboratory for this purpose.

The procedure which is described in this paper has been used successfully to measure LH in blood throughout the oestrous cycle, during pregnancy, the post partum period and nutritional anoestrus in cows of various breeds under local conditions of management (Hale, 1974b). Additionally, this procedure has been used to assess the production of LH by the bovine anterior pituitary *in vitro* and to study the relationships between this production and the activity of the pineal-hypothalamic axis (Hayes, Knight & Symington, 1974; Symington, Hayes, Knight & Hale, 1973).

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