

Solid-phase Assay for Luteinizing Hormone in Mouse Plasma

A. A. Gidley-Baird^A and B. M. Bindon^B

^A Department of Veterinary Physiology, University of Sydney, Sydney, N.S.W. 2006.

^B Division of Animal Genetics, CSIRO, P.O. Box 90, Epping, N.S.W. 2121.

Abstract

A solid-phase tube assay for measuring LH levels in mouse plasma is described. The assay utilizes an antiserum to ovine LH and ovine LH standards and it measures LH levels in 20 μ l of plasma with a sensitivity of less than 0.6 ng/ml. Various parameters affecting the sensitivity and specificity of the assay were investigated. Serial dilutions of plasma from pregnant mice, a pituitary homogenate from mice and plasma from hypophysectomized mice, injected subcutaneously with ovine LH, ran parallel with ovine LH standards in plasma from hypophysectomized mice and plasma with low LH levels from intact mice. Ovine TSH showed about 12% cross reaction in the assay system, whilst rat FSH and prolactin and also ovine FSH, prolactin and GH showed practically no cross reaction. Measurements of plasma LH levels have been made in hypophysectomized mice after injection with different vehicles containing 10 or 50 μ g LH or 50 μ g FSH per animal. Daily measurements of LH levels throughout pregnancy in the mouse show a rise in LH level prior to implantation and a further rise around mid-pregnancy which drops off sharply to levels which remain fairly constant until parturition when there is another rise.

Introduction

The published immunoassays for mouse LH (Beamer *et al.* 1972; Kovacic and Parlow 1972) require 100 and 300 μ l of sample plasma respectively. This volume does not permit concomitant measurement of the levels of several hormones or repeated measurements of one hormone from the same animal. Both these assays utilize a double-antibody technique and thus lack the practical advantages of time saving and simplicity which are available with the use of a solid-phase system. This paper describes a sensitive solid-phase method for mouse plasma based on the assays of Catt (1969) and Goding *et al.* (1969).

In this solid-phase method LH measurements in plasma from hypophysectomized mice were made at different time intervals after subcutaneous injection of LH in different vehicles, and after injections of FSH. A comparison was made between LH levels in plasma collected from the tail vein and in plasma collected after decapitation of the same animal. Daily measurements were made throughout pregnancy in the mouse.

Materials and Methods

Antiserum to LH

The antiserum (ALH) was raised in a rabbit against ovine LH (NIH LH S-16). This antiserum, when injected subcutaneously into pregnant mice before 1800 h on day 3 of pregnancy (day 1 is the day that the vaginal copulatory plug is found), will prevent implantation from occurring.

Radioimmunoassay (RIA) Diluent

The diluent was phosphate buffer (0.05M, pH 5.6) containing EDTA (0.01M), merthiolate (0.01%), crystalline egg albumin (0.01%, Selby and Co., Australia) and 15% serum from an anaestrous bitch. The diluent was filtered through a Whatman No. 1 filter prior to use.

Iodination of Pituitary Hormones

The chloramine-T method of Greenwood *et al.* (1963) was used to iodinate 10 μg of NIH LH S-16 with 2 mCi of Na^{125}I (Radiochemical Centre, Amersham, England). The reaction utilized 100 μg of chloramine-T and was terminated after 1 min by adding 120 μg of sodium metabisulphite. Separation of iodinated LH from free ^{125}I was performed on a 6 by 0.5 cm column of cellulose (Whatman CF11) from which the labelled LH was eluted with a solution of 20% acetone in 0.02M barbitone buffer, pH 8.6, containing 5% bovine serum albumin (Sigma fraction V) and 0.02% merthiolate. For FSH, 10 μg of Sherwood ovine FSH (133 \times NIH FSH S-1) was iodinated in a similar manner, the only change being the use of 80 μg of chloramine-T reacted for 1.5 min before the addition of sodium metabisulphite.

Hormones

The hormones used in this study are as shown in the following tabulation:

LH	NIH ovine LH S-16	Prolactin	NIAMD rat prolactin RP-1
FSH	NIAMD rat FSH B-1	TSH	NIH ovine TSH S-6
	NIAMD rat FSH RP-1	GH	NIH bovine GH B-17
	NIH ovine FSH S-8	HCG	Pregnyl, Organon
	Sherwood ovine FSH (133 \times NIH FSH S-1)		

A crude homogenate of mouse pituitary was made by taking 50 pituitary glands from pregnant mice on days 2 and 3 of pregnancy. These glands were homogenized in cold 0.9% NaCl (w/v) solution and the mixture was then filtered on a Whatman No. 1 paper and stored at -25°C until used.

Mice

Quackenbush strain adult female mice aged between 8 and 10 weeks and weighing 25–30 g were used. The animals were hypophysectomized using the Bindon and Lamond (1969) modification of the method of Lamond and Emmens (1959). After the operation the mice were maintained on normal pelleted laboratory food and given 5% glucose in their water.

Collection of Mouse Plasma

For assay analysis, blood was collected by decapitating the animal or by cutting of a small piece of the tail and collecting blood into preheparinized 50- μl microhaematocrit tubes (Hawksley). Hypophysectomized mouse plasma was obtained by removing the pituitary glands from adult female mice and killing them by decapitation 2 days later. The blood was pooled and centrifuged, the plasma then drawn off and stored in aliquots at -25°C .

Analysis of Data

The data from the radioimmunoassay were analysed using the program of Rodbard and Lewald (1970). This was converted to Fortran by Dr N. J. Jackson, Division of Animal Genetics, CSIRO. To test for parallelism between two or more sets of displacement responses in the one assay, the weighted regression coefficients given for them, using the above program, were tested to see whether they differed significantly.

The data for LH levels during pregnancy in the mouse, obtained by repeated sampling of individual mice, were subjected to an analysis of variance using the program FACANOVA designed for use on the CYBER 7200 at the University of Sydney (I. C. A. Martin, personal communication). The variance was partitioned using a matrix of orthogonal coefficients up to the power 20.

Investigation of Factors Affecting the Assay of Mouse LH

Experiment 1. pH Effects

This experiment studied the effect of changes in pH on the adsorption to the tubes of antisera diluted in tris-NaCl buffer and the binding and displacement properties of the assay with changes in pH for the RIA diluent and for the pH of the buffer in which the label was added.

- (1) Various dilutions of antisera in tris-NaCl buffer at pH 9.6, 7.8 and 5.8 were incubated in tubes for 2 h. The contents of the tubes were drawn off and the tubes rinsed. RIA diluent and labelled LH were added, the tubes incubated at 37°C for 24 h and the counts bound to the tubes measured.
- (2) Based on the results of the procedure outlined in (1), displacement with standards of 0.1, 1.0 and 10.0 ng of LH (NIH S-16) was carried out with the antiserum adsorbed at dilutions of 1 : 80 000 and 1 : 120 000 from tris-NaCl buffer at pH 7.8 and 9.6.
- (3) With the antiserum adsorbed to tubes at 1 : 80 000 in tris-NaCl, pH 7.8, displacement with standards of 0.1, 1.0 and 10.0 ng of LH was carried out with RIA diluent at pH 5.6, 6.6, 7.8 and 8.6.
- (4) With the antiserum adsorbed at 1 : 120 000 in tris-NaCl, pH 7.8, two sets of standards for LH were run in RIA diluent, pH 5.6, with the labelled LH added in 0.05M phosphate buffer at pH 5.6 and 7.5.

Results

Fig. 1a shows that at high concentrations of the antiserum a pH of 9.6 was more effective in causing antibody adsorption to the tubes but at lower concentrations of antiserum incubation at pH 7.8 was more effective. Fig. 1b supports these findings and shows a greater relative displacement over the range of standards with both dilutions of antibody incubated at pH 7.8. Fig. 1c demonstrates that incubation in RIA diluent at pH 5.6 gave increased binding and more effective relative displacement. There was little difference in the displacement curves for the labelled LH added in buffers at pH 5.6 and 7.5 (Fig. 1d).

Experiment 2. Plasma and Serum for Standards and Diluent

This experiment studied the addition of LH standards in plasma from several species and the use of plasma or serum from different species in the diluent. The plasma used came from the hypophysectomized female mouse, rat, rabbit, wallaby; intact human male and anoestrous bitch.

Results

The results showed that, although the standards when run in different plasmas gave displacement curves that were parallel, there was a difference in binding affinity of the labelled LH due to plasma differences. The differences shown for the rabbit and wallaby plasmas appeared to be due mainly to the increase in non-specific binding whereas the rat and intact human male plasmas appeared to contain some level of LH which cross reacted in the system. There was only a very small difference between using 20 μ l of serum or plasma for addition of the standards but in the diluent, despite a higher level

of non-specific binding, there was an increase in binding affinity and relative displacement using serum instead of plasma. Using 15% anoestrous bitch serum in the diluent instead of 10% hypophysectomized mouse plasma gave an increased relative displacement with the same low level of non-specific binding.

Experiment 3. Temperature and Incubation Time

The effect of temperature was examined by running the assay at 3, 23 and 37°C and optimum incubation time was assessed by varying the length of the first and second incubation steps.

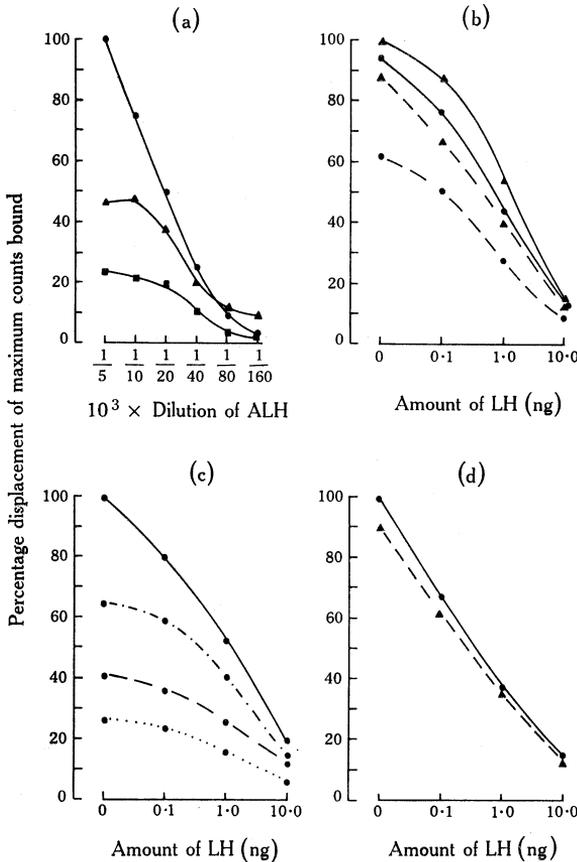


Fig. 1. (a) Effect of pH on the adsorption of antibody to the solid phase from tris-NaCl buffer at pH values of 9.6 (●), 7.8 (▲) and 5.8 (■). (b) Displacement curves for NIH LH S-16 with antibody dilutions of 1:80000 (—) and 1:120000 (---) adsorbed at pH values of 9.6 (●) and 7.8 (▲). (c) Effect of the pH of RIA diluent on the displacement of NIH LH S-16 in the assay. Diluents of pH 8.6 (⋯), 7.6 (---), 6.6 (---) and 5.6 (—) used. (d) Effect of the pH of the buffer from which labelled LH was added on the displacement of NIH LH S-16 in the assay. Buffers of pH 7.5 (●) and 5.6 (▲) used.

Results

The incubation at 37°C gave a greater binding affinity and a larger relative displacement over the range of standards tested. A 24-h first incubation with a 24-h second incubation gave the optimum conditions. Periods shorter than this gave a reduction in the number of displaceable counts bound and longer times gave a considerable increase in non-specific binding.

Experiment 4. Assay Validity and Sensitivity

For the assay to be valid it was necessary to show that mouse LH in both the pituitary and plasma behaved, in this assay, in the same manner as the ovine LH

standards. It was also of interest to know whether ovine LH showed any altered assay characteristics after injection and absorption into hypophysectomized mice and whether it behaved differently if assayed in hypophysectomized mouse plasma or intact mouse plasma.

Sensitivity is defined as the smallest quantity of LH that can consistently be detected, in a fixed volume of plasma, by the assay system. To allow repeated samples to be taken from the same mouse, LH was routinely measured in 20- μ l samples of plasma but for the purpose of testing the sensitivity of the assay, LH was measured in 100- μ l samples. Sensitivity (expressed as nanograms of LH per millilitre of plasma) and validity of the assay were examined as follows:

- (1) Ovine LH (NIH LH S-16) standards in 0.1 ml of hypophysectomized mouse plasma were run against serial dilutions of a crude homogenate of mouse pituitary gland and serial dilutions of plasma collected from female mice killed at 0800 h on day 4 of pregnancy. The dilutions of pituitary gland homogenate and pregnancy plasma were made with the plasma from the pool of hypophysectomized mouse plasma used for the standards.
- (2) Ovine LH standards in 0.1 ml of hypophysectomized mouse plasma were run against serial dilutions of plasma from female mice hypophysectomized on day 2 of pregnancy, injected 2 days later with 50 μ g of NIH LH S-16 suspended in a 15% gelatin vehicle and then killed 2 h after the injection.
- (3) Ovine LH standards in 20 μ l of hypophysectomized mouse plasma were run against LH standards suspended in plasma from a pool collected from female mice killed 24 h post-coitus.

Results

Fig. 2a shows that serial dilutions of day 4 pregnancy plasma and of crude homogenate of mouse pituitary gland run parallel with the displacement curve for the LH standards. The regression coefficients and standard deviations are 911.3 ± 67.1 , 862.6 ± 96.7 and 857.4 ± 81.8 respectively (no significant differences at the 5% level). Fig. 2b shows that the displacement curve for dilutions of plasma from hypophysectomized mice injected with LH runs parallel with the curve obtained for the same LH used for standards and suspended in hypophysectomized mouse plasma. The regression coefficients and standard deviations are 134.1 ± 26.9 and 129.9 ± 26.4 respectively. Fig. 2c shows that there is little difference between the displacement curve for the LH standards in hypophysectomized mouse plasma and the displacement curve for the LH standards in pregnancy plasma containing low levels of endogenous LH.

Experiment 5. Assay Specificity

Assay specificity was examined by comparing the displacement of sets of the following gonadotrophin standards:

- (1) LH, NIH S-16; FSH, NIH S-8 and NIAMD rat RP-1; TSH, NIH S-6; prolactin, NIAMD rat RP-1; GH, NIH B-17: these hormones were assayed at levels of 0.1, 1.0, 10.0 and 100.0 ng in 20 μ l of hypophysectomized mouse plasma. HCG (pregnyl, Organon) was added in 20 μ l of hypophysectomized mouse plasma at levels of 0.1, 1.0, 10.0 and 100.0 i.u. Iodinated LH (NIH LH S-16) was used as the tracer.

(2) The above procedure was repeated using iodinated FSH (Sherwood ovine FSH) as the tracer.

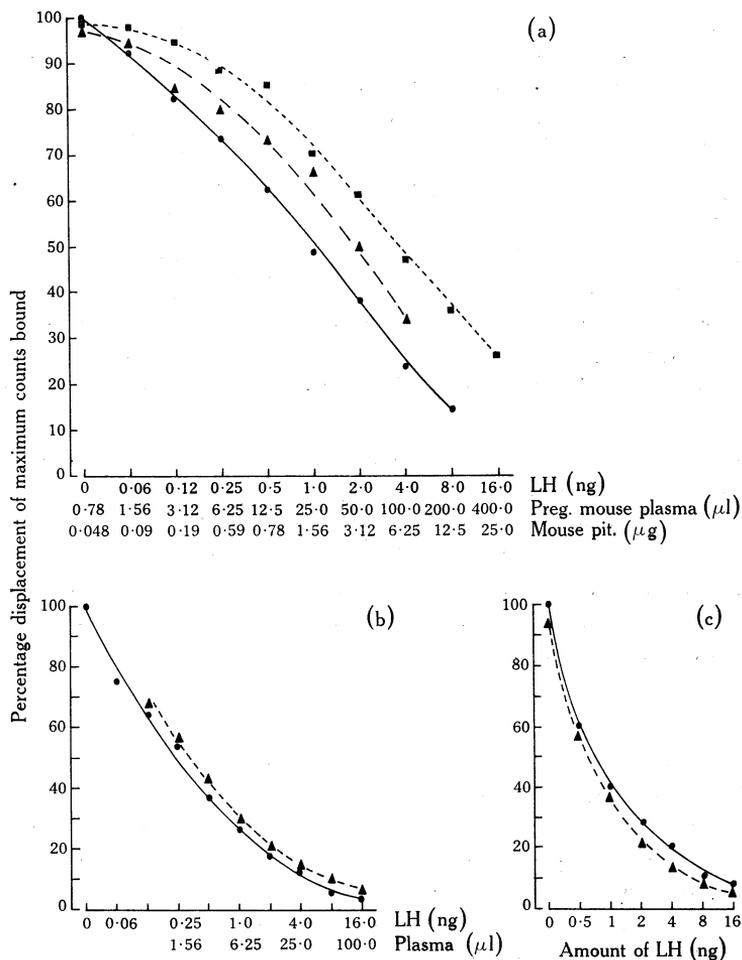


Fig. 2. (a) Comparison between NIH LH S-16 (\bullet), homogenate of mouse pituitary gland (\blacksquare) and plasma of pregnant mouse (\blacktriangle) in the capacity to displace labelled NIH LH S-16 in the assay. (b) Comparison in the assay of the displacement of NIH LH S-16 added directly to hypophysectomized mouse plasma (\bullet) and of a serial dilution of plasma from hypophysectomized mice injected with NIH LH S-16 (\blacktriangle). (c) Comparison of NIH LH S-16 standards added to hypophysectomized mouse plasma (\bullet) and added to plasma from female mice killed 24 h post-coitus (\blacktriangle).

Results

Ovine or rat FSH caused very little displacement of labelled LH from the antiserum and most of the displacement can be accounted for by the stated LH contamination of the preparations. TSH, however, did show a considerable displacement in the assay system and was approximately 12% as effective as LH. HCG showed displacement and was about 4% as effective as LH whilst labelled Sherwood FSH bound to the

antisera and was about 11% as effective as the labelled LH. The displacement of labelled FSH in the assay by LH, TSH, HCG, and ovine and rat FSH showed a displacement pattern very similar to that found when using labelled LH as the tracer. Prolactin and GH showed practically no displacement in the assay.

Radioimmunoassay Protocol

The protocol described here is the working procedure arrived at after experimental investigation of factors and reagents that could influence the assay.

- (1) 0.8 ml of ALH diluted to 1 : 65 000 in a tris-NaCl buffer, pH 7.8, was added to polystyrene tubes (75 by 10 mm, BSS30003 Camelec, Australia). Three tubes containing the same dilution of normal rabbit serum were included to give an estimate of non-specific binding for each assay. This value is routinely subtracted from all values before analysis of the data. All tubes were kept at room temperature for 2 h, the contents were then drawn off by suction and the tubes rinsed once with a solution of 0.9% (w/v) NaCl.
- (2) RIA diluent (0.8 ml) was added to all tubes. Standards of NIH LH S-16 were added in 20 μ l of plasma from hypophysectomized mice. Plasma unknowns were added to tubes as 20- μ l samples. Triplicates of all standards and unknowns were included. The three tubes incubated with normal rabbit serum received 20 μ l of hypophysectomized mouse plasma. All tubes were mixed briefly on a vortex mixer and incubated in a water bath at 37°C for 24 h.
- (3) ¹²⁵I-labelled LH (specific activity 200 μ Ci/ μ g) was diluted in 0.05M phosphate buffer, pH 7.5, and approximately 50 000 cpm, equivalent to 0.5–0.6 ng of LH, was added to each tube. The tubes were spun on a vortex mixer and incubated for a further 24 h at 37°C.
- (4) The contents of the tubes were then drawn off by suction, the tubes rinsed twice with 0.9% NaCl solution and counts were made on a Packard auto-gamma counter to a minimum of 10 000 counts.

Assay Characteristics

Using the protocol described and running the assay within a week of labelling the LH, 6000–7000 cpm were bound in tubes containing hypophysectomized mouse plasma with no LH (zero LH tubes). Between 60 and 70% of the counts bound in the zero LH tubes were displaced by 1.0 ng of NIH LH S-16. The non-specific binding was typically between 100 and 200 cpm. Replicate reliability typically showed a variation around 5% or less. The mean and standard deviation for the index of precision (λ) for 10 assays was 0.049 ± 0.021 . The coefficient for the variation of estimate of LH values for samples from a pool of plasma run in 10 different assays was 9.4%. The sensitivity of the assay was 0.6 ng/ml of plasma; this could easily be altered by varying the concentration of antiserum bound to the tube or the amount of labelled LH added per tube or both.

Measurement of LH Levels after Injection into Hypophysectomized Mice

In this study levels of plasma LH were measured in hypophysectomized mice injected with LH or FSH in different injection vehicles. LH levels were also compared in

plasma samples taken from the tail vein and plasma samples taken after decapitation of the same animal.

Adult female mice were hypophysectomized and 2 days later received subcutaneous injections as follows:

- (1) All animals in three groups of 25 mice received 50 μg of NIH LH S-16. The first group received the LH in 0.1 ml of saline, the second in 0.1 ml of 15% gelatin solution and the third in 0.1 ml of sesame seed oil.
- (2) All animals in two groups of 25 mice were injected with 10 μg of NIH LH S-16. The first group received the LH in 0.1 ml saline, the second group in 0.1 ml of a 15% gelatin solution. This last group had blood samples taken from the tail vein just prior to decapitation.
- (3) Two groups of 15 mice received 50 μg of NIAMD rat FSH B-1; one group received the FSH in 0.1 ml saline and the other group in 0.1 ml of 15% gelatin solution.

Five animals in each group of (1) and (2) were killed at intervals of 0.5, 1, 2, 4 and 8 h after injection. Five animals in each group of (3) were killed 1, 4 and 8 h after injection. All plasma samples were assayed for LH content using the assay protocol described.

Results

Figs 3*a-c* show the mean and standard deviation of plasma LH levels for five mice killed at each time interval after having received 50 μg of LH in different injection vehicles. The 15% gelatin vehicle clearly maintains a more uniform level of LH for a longer period (Fig. 3*b*). Figs 3*d-f* show the mean and standard deviation of plasma LH levels for five mice killed at each time interval after having received 10 μg of LH in saline or 15% gelatin. They also show a comparison between levels obtained from samples taken from the tail vein and samples taken after decapitation. The results show that there is a slower and more uniform absorption from the gelatin vehicle and that plasma LH levels obtained by sampling from the tail vein are slightly but not significantly lower than those obtained after decapitation. Only in the case of FSH in gelatin and the animal killed 1 h after injection was a value for LH recorded above the limit of sensitivity of the assay, the mean value being 1.0 ng/ml.

Measurement of Daily Plasma LH Values Throughout Pregnancy

(i) LH values in pooled plasma

Five mice were killed at 0900 h for each day of pregnancy and 0.2 ml of plasma was collected from each mouse and stored at -20°C until assayed. On the day of assay all the plasma from each time interval was pooled and filtered through Whatman No. 1 filter paper. Each plasma pool was assayed in quadruplicate. The pregnant mice used in this part of the experiment came from three groups of mice that showed copulatory plugs on the same day. On two days there were enough mice to allocate two mice for plasma samples on each day of pregnancy, the other day gave enough to assign one animal to be killed each day.

(ii) *LH values from daily repeated tail vein bleedings in individual mice during pregnancy*

Preliminary studies showed that daily bleedings of mice for a prolonged period did in some cases adversely affect the pregnancy. Accordingly the following protocol was adopted. Mice that showed copulatory plugs on the same day were divided into three groups. Daily samples were taken from the first group on days 1–8 of pregnancy. Samples from the second group were taken on days 7–15 and from the third group on days 14–20. All samples were taken between 0900 and 1000 h. All the mice were allowed to go to term and the plasma from any mouse that did not litter was not assayed and was therefore not included in the results.

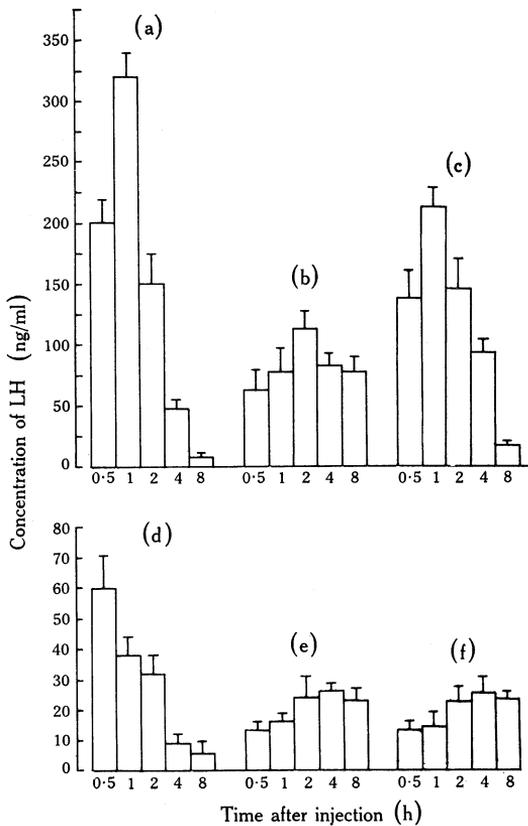


Fig. 3. Plasma levels of NIH LH S-16 in groups of hypophysectomized mice at different time intervals after injection of 50 μg of LH (a-c) and 10 μg of LH (d-f) administered in different vehicles. Standard errors are shown as vertical bars. (a) Saline, (b) 15% gelatin, (c) sesame seed oil, (d) saline, (e) 15% gelatin, plasma collected after decapitation, (f) 15% gelatin, plasma collected from the tail vein of animals used in (e) just prior to decapitation plasma collection.

(iii) *Results*

Fig. 4 shows daily plasma LH levels in the mouse during pregnancy. Both of the techniques used for collecting the plasma samples show the same basic pattern for LH values. The LH level is low on days 1 and 2, rises prior to implantation which occurs late day 5–early day 6, then remains steady until a further rise occurs starting on day 9 and giving the highest LH values on days 11 and 12. After day 12, the LH level drops sharply and remains fairly constant until around the time of parturition (day 19) when it rises.

An analysis of variance using the data from the repeated sampling technique and the program FACANOVA showed a significant variation ($P < 0.01$) in plasma LH levels during pregnancy. Partitioning of the variance to the power 20 using orthogonal coefficients revealed that the data significantly fitted ($P < 0.05$) linear, quadratic and cubic equations, the cubic equation indicating two significant points of inflection in the description of the data. From the data it is apparent that the analysis indicates a rise to a peak at mid-pregnancy followed by a sharp decline in LH values and then a rise occurring around parturition.

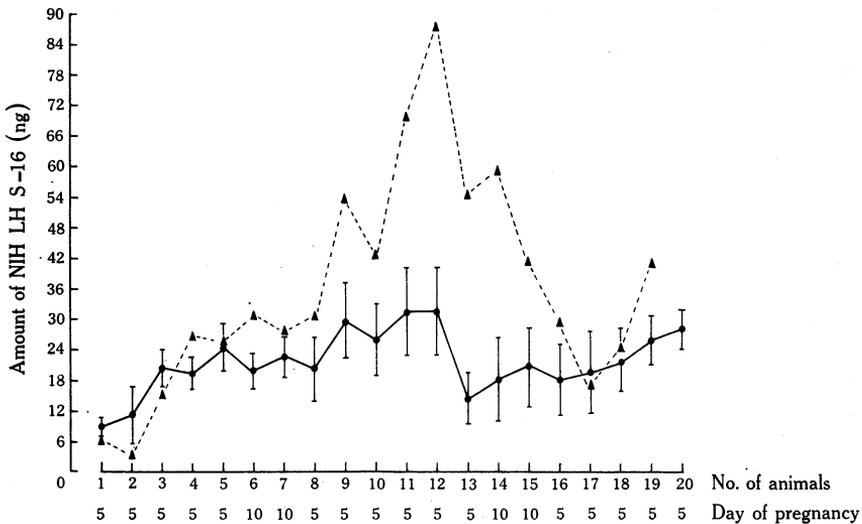


Fig. 4. Daily plasma LH values throughout pregnancy in the mouse. ▲ LH values for pooled plasma samples. ● Mean LH values (\pm S.D.) for plasma samples collected by repeated tail vein bleedings. The number of mice bled at each time interval for repeated bleeding samples is shown under the day of pregnancy.

Discussion

This work demonstrates that a solid-phase assay for LH using an antiserum raised against ovine LH and ovine LH standards can be used for the measurement of LH levels in mouse plasma. Conditions have been described which give the assay a sensitivity such that it can be used for making repeated measurements of plasma LH levels from a single mouse. The finding in this assay system that iodinated Sherwood ovine FSH is bound about 10% as effectively as labelled LH by ALH and is displaced by LH and not FSH is difficult to explain. The immediate response is to assume that this FSH preparation contains some LH contamination. However, Sherwood *et al.* (1970) reported a very much lower immunoreactive LH contamination than these results indicate.

For the purpose of replacement therapy experiments one should ascertain the normal physiological hormone levels by direct measurement and approximate these as closely as possible. In endeavouring to mimic these normal levels account should be taken of the replacement dose with the absorption characteristics of the vehicle in which the hormone was injected. The experiment in which plasma LH levels were

measured in hypophysectomized mice after injection of LH in different vehicles shows that plasma LH was maintained at a more constant level for a longer period of time when injected in a 15% gelatin solution than when injected in saline or oil. Plasma samples collected from the tails of mice gave slightly but not significantly lower LH values than plasma samples collected after decapitation. Taking small samples of plasma from the tail gives a means of collecting repeated samples from one animal and thus eliminates between animal variation when monitoring changes over a period of time.

The LH levels recorded by daily measurements throughout pregnancy of the mouse agree well with the values reported by Murr *et al.* (1974). The pre-implantation rise in LH level and the mid-pregnancy LH peak reported by these authors were confirmed in this study. The data reported here, however, do differ in one respect from that of Murr and coworkers. They reported a drop of LH level to very low levels on days 5–8 after the pre-implantation rise and again on days 13–15 after the mid-pregnancy peak. The results presented here did not show a fall in LH level after the pre-implantation rise and although there was a significant decline in LH level after the mid-pregnancy peak the levels recorded on days 13–15 were not lower than those found on day 3. This may reflect a strain difference, as the basic pattern of plasma LH levels appears the same. The mid-pregnancy rise in LH level occurred in all individual mice subjected to repeated sampling at this time. As this rise occurred over the range of 3 days, depending on the individual animal, the way the data have been presented tends to show a broad rise with large standard deviations instead of the sharp rise that does in fact occur. This rise in LH level occurs at a time when the pituitary is no longer essential for the maintenance of pregnancy and coincides with the time of a sharp rise in LH level which causes ovulation and terminates pseudopregnancy in the pseudopregnant mouse (Kovacic and Parlow 1972). The fact that the rise occurs in both pregnant and pseudopregnant mice suggests that it is part of an endogenous pituitary rhythm of the mouse and is not of placental origin; a possibility suggested because of the autonomy of the ovarian-foetal unit from the pituitary which occurs at this time.

Acknowledgments

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