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A MICRO-RADIOIMMUNOASSAY FOR MEASUREMENT OF RAT LUTEINIZING HORMONE

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OPSOMMING: 'N MICRORADIOIMMUNO-BEPALINGSMETODE VIR DIE BEPALING VAN ROT LH

'n Sensitiewe dubbel teëliggaam radioimmuno-bepalingsmetode vir rot LH wat maksimaal 50 μℓ serum of plasma per buisie benodig word beskryf. Die metode gebruik ^{125}I en konyn anti-rot LH serum. Die bepalingsmetode het 'n sensitiwiteit van 16 ng LH/mℓ en koëffisiente van variasie tussen en binne bepalings van ± 6,6% en ± 3,1% respektiewelik. Die moontlike diffusie van die bestandele deur die wande van plastiek microbuisies gedurende inkubasie was nie betekenisvol. Normale basale LH waardes van albino rotte tesame met die serum LH reaksies op verskillende dosisse Luteïeniserende hormoon vrystellings hormoon (LVH) word beskryf.

SUMMARY:

A sensitive and reproducible double antibody radio-immunoassay for rat LH measurement, which requires at most 50 μℓ of serum or plasma per tube, is described for use with ^{125}I and rabbit anti-rat LH serum. The assay has a sensitivity of 16 ng LH/mℓ, and coefficients of inter-assay and intra-assay variations of ± 6,6% and ± 3,1% respectively. The possible diffusion of the constituents of the incubate through the walls of the plastic micro tube used, as described by others, was not significant. Normal basal LH values for albino rats are described together with their serum LH responses to various doses of LRH.

Sequential blood sampling from small laboratory animals for several hours necessitates the collection of small volumes of blood which can therefore be assayed only by micro techniques. Such methods have been developed for, among others, insulin (Weinkove, Weinkove & Pimstone, 1974) and luteinizing hormone (LH) (Naftolin & Corker, 1971). The availability of purified rat pituitary hormones and antibodies through the National Institutes of Health in America, has made it possible to establish a micro immuno-assay for rat LH in this laboratory. The purpose of this paper is to describe the characteristics and reliability criteria of this assay and to define normative data for LH levels in our rat colony. A report of this work in abstract form was published recently (Querido & Beardwood, 1975).

Materials

Buffers:

0,5M Phosphosaline Buffer (0,5M PSB); 0,15M NaCl, 0,5M Na_2HPO_4 – pH adjusted to 7,5 by addition of 0,5M KH_2PO_4 in 0,15M NaCl.

0,01M Phosphosaline Buffer (0,01M PSB); 1/50 dilution of 0,5M PSB with 0,15M NaCl – pH 7,4.

0,01M Phosphosaline Buffer containing 0,002M ethylenediaminetetraacetic acid (0,01M PSB EDTA) – pH 7,6.

Hormones and Antisera:

Standard LH: (NIAMD-Rat LH-RP-1). Diluted in 0,01M PSB containing 0,5% HSA such that 1 mℓ contained 1 μg of LH. Stored at –18°C in 1 mℓ aliguots.

Purified LH for labelling: (NIAMD-Rat LH-1-1). Diluted with 0,01M PSB such that 10 μℓ contained 2,5 μg of LH. Stored at –18°C in 15 μℓ aliquots.

LH Antiserum: (NIAMD-Anti-Rat LH Serum-l) "First Antibody". Dispensed in 0,1 mℓ aliquots as a 1/100 dilution with 0,01M PSB containing 1% Normal Rabbit Serum (NRS) and stored at –18°C.

Precipitating Antiserum: "Second Antibody" or Anti-rabbit gamma globulin (ARGG). (Anti-Rabbit Precipitating Serum (donkey), Wellcome Reagents LTD.)

Iodination Reagents:

Chloramine-T (E. Merck AG Dermstadt)
12,5 mg in 5 mℓ 0,01M PSB (prepared immediately before use).

Sodium Metabisulphite (May and Baker LTD)
10 mg in 5 mℓ 0,01M PSB (prepared immediately before use).

Sodium ^{125}Iodide (Radiochemical Centre, Amersham) 500 μCi contained in 5 μℓ.

Sephadex G-50 fine (Pharmacia Fine Chemicals LTD) Prepared in 0,01M PSB.

CF 11 Fibrous Cellulose Powder (Whatman Column Chromedia). Prepared in 0,01M PSB.

Other Reagents:

Normal Rabbit Serum (NRS) – Calbiochem.
Human Serum Albumin (HSA) – Natal Blood Transfusion.
LH-releasing hormone (LRH) – Ayerst Laboratories.

Animals:
Albino rats of a mixed strain exposed to light between 06h00 and 20h00, were used throughout the study. Blood samples were collected between 09h00 and 10h00 from conscious unrestrained animals through a chronic indwelling jugular cannula. The cannula was placed, with slight modifications (Querido, 1975) according to to the technique of Steffens (1969).

Method

The assay, which extends over a period of 11 days, was performed in duplicate in capped microtubes (Thomas Micro Centrifuge Tubes – Thomas Scientific Apparatus). With the exception of second antibody which was added together with NRS in 100 μℓ volumes, all the other reagents and the unknown samples were added as 50 μℓ aliquots. The assay tubes, each containing a final volume of 250 μℓ, were arranged in duplicate as shown in Table 1. The day-to-day assay procedure was as follows:

Day 1 (am): The following reagents were added to the assay tubes: A 1 in 64 000 dilution of first antibody in 0,01M PSB EDTA containing 4% HSA to all tubes other than the total count (TC) tubes and non-specific binding (NSB) tubes; rat LH standards in 0,01M PSB varying between 4 and 500 μg LH/mℓ to tubes labelled 1 to 16 (Tabel 1); the unknown samples were added to tubes labelled "NSB unknown" as an index of non-specific binding of label to these samples. This procedure of obtaining "NSB unknown" was repeated for each batch of 10-12 plasma samples. Buffer was added to bring the volume in each tube to 100 μℓ. In addition, tubes containing 50 μℓ of pooled rat serum were included in each assay as a check on the accuracy and reproducibility of the assay. After vortex mixing and centrifugation to remove the air bubbles, the tubes were incubated at 4°C for 3 days.

Day 4 (am): Iodination of rat LH was carried out according to the Chloramine-T method of Hunter and Greenwood (1962). The reaction was performed in an autoanalyzer vial (Sterilin LTD) and the reactants added in the following sequence:
$Na^{125}I$ – 500 μCi (5 μℓ); 0,5M PSB (20 μℓ); purified LH–2,5 μg (10 μℓ); and Chloramine-T–25 μg (10 μℓ). A reaction time of 25 seconds elapsed before addition of 200 μg sodium metabisulphite (100 μℓ). After separation of the labelled LH from the free ^{125}I on a Sephadex G-50 column (5 x 100 mm) eluted with 0,01M PSB, the labelled LH was further purified using a cellulose CF 11 column (5 x 50 mm) eluted first with 18 mℓ of 0,01M PSB and then with the same buffer containing 5% HSA. The specific activities of the labelled hormone varied between 35 and 70 μCi/μg. The radioactive LH was diluted to about 20 000 cpm/50 μℓ and added to each assay tube. After mixing and centrifuging as before, the tubes were incubated at 4°C for 6 days.

Day 10 (am): The second antibody solution (ARGG) was prepared at a 1 in 32 dilution in 0,01M PSB EDTA containing 0,5% NRS.

Table 1

Volumes of the different solutions contained in the rat LH assay tubes.

LH Concentration ng/mℓ	Tube	Buffer	Antibody*	LH STD**	Labelled LH*	NRS & ARGG
	TC	–	–	–	50 μℓ	–
	NSB	50 μℓ* 50 μℓ **	–	–	50 μℓ	100 μℓ
0		50 μℓ **	50 μℓ	–	50 μℓ	100 μℓ
4	1– 2	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
8	3– 4	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
16	5– 6	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
32	7– 8	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
62,5	9–10	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
125	11–12	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
250	13–14	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
500	15–16	–	50 μℓ	50 μℓ	50 μℓ	100 μℓ
NSB unknown		50 μℓ	–	50 μℓ serum pool	50 μℓ	100 μℓ
Unknown serum		–	50 μℓ	50 μℓ serum	50 μℓ	100 μℓ

* 0,01M PSB EDTA containing 4% HSA
** 0,01M PSB

$100 \mu \ell$ of this solution was added to each tube, and the contents of the tubes were mixed, centrifuged briefly and incubated at 4°C for 24 hours.

Day 11: All tubes other than TC tubes were centrifuged at 3000 rpm for 30 minutes. Holding the tip of each tube, it was flicked once to force the supernatent fluid towards the capped end. The tip of each tube, containing the precipitate, was cut off and its radioactivity measured in a gamma counter.

Determination of the LH concentrations from the standard curve and measures of reliability criteria were carried out according to standard procedures (Midgley, 1969).

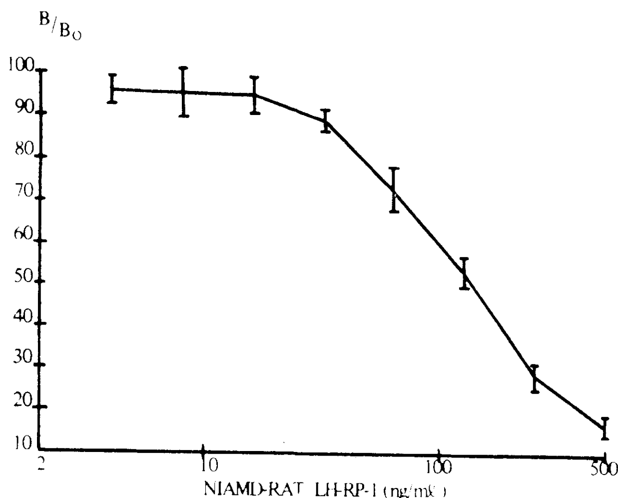
Results

Reliability Criteria

The sensitivity of the assay, defined as the least amount of hormone which can be distinguished from no hormone (Midgley, 1969), was 16 ng LH/mℓ, since its corresponding mean B/Bo value (94,6 ± 3,8%) derived from 5 consecutive assays was significantly different from that of the zero-point ($P < 0,05$) while the difference between the zero-point and the 32 ng LH/mℓ (88,7 ± 2,4%) was highly significantly different ($P < 0,001$).

The reproducibility of the technique was estimated by measuring LH levels in the same samples in a series of consecutive assays (inter-assay variation) and by measuring LH levels in replicate samples in a single assay (intra-assay variation). Mean B/Bo values (± SD) derived from 5 consecutive standard curves are shown in Fig. 1.

Fig. 1. *LH inhibition curve showing mean B/Bo values (expressed as a percentage) (± SD), at each point.*



The mean coefficient of inter-assay variation, using all points along the curve, was ± 6,6%, while the determination of inter-assay variation using pooled serum specimens yielded a value of ± 6,4%. Mean B/Bo values (± SD) corresponding to 5 replications at four points along the standard curve (inter-assay variation) are shown in Table 2. The mean coefficient of intra-assay variation over this portion of the curve was found to be ± 3,1%.

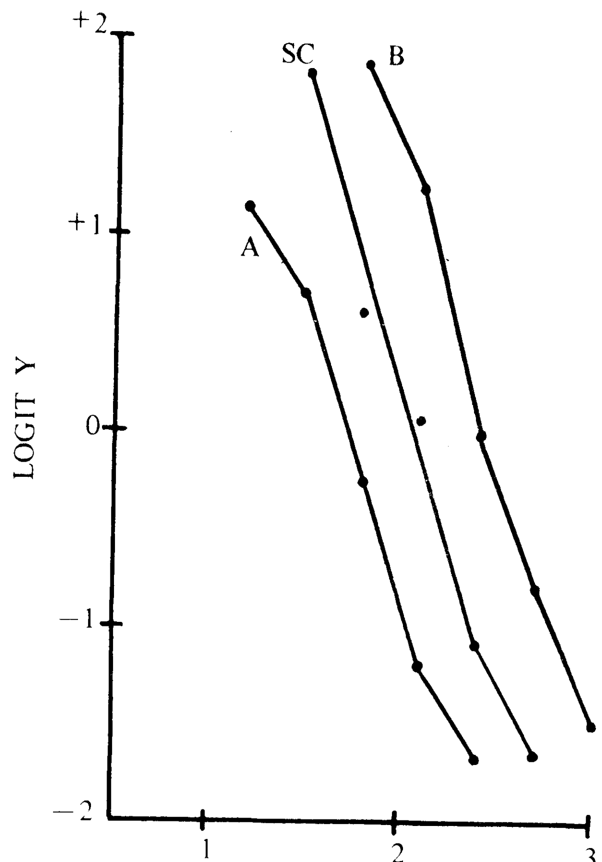
Table 2

%B/Bo values for five replicates of four different LH levels estimated within one assay.

	LH Concentration (ng/mℓ)			
Sample No.	32	62,5	125	250
1	90,0	79,4	58,9	31,4
2	89,6	76,1	56,4	33,6
3	90,5	75,4	56,9	32,1
4	88,1	77,7	59,5	36,5
5	93,1	76,3	57,1	34,3
Mean % B/Bo	90,3±	77,0 ±	57,7±	33,6±
± SD	1,8	1,6	1,4	2,0

Finally, in order to test for comparable immunological behaviour between LH in the unknown serum samples and standard LH over a wide range of hormone levels, doubling dilutions of two serum samples otbained from ovariectomized rats, were prepared in 0,01M PSB containing 4% HSA and their LH levels measured. B/Bo values plotted on the same graph as the standard curve are shown after their transformation to the logit notation (Midgley, 1969) (Fig. 2). The serum samples show good parallelism with the standard curve.

Fig.2 *Logit transformation of inhibition curves which demonstrate parallelism between standards (SC) and unknown serum samples (A & B).*



Radioactive Leakage

Two experiments were conducted to measure possible loss of radioactivity from the plastic microfuge tubes used in the assay. In the first experiment 250μℓ of labelled LH solution and in the second 250μℓ of $Na^{125}I$ solution was added to the microfuge tubes. The latter were placed in glass test tubes containing 1,0 mℓ PSB. Half the tubes in each experiment were incubated at 4°C, while the other half were incubated at room temperature. The radioactivity of the contents of both glass and plastic tubes was measured daily for 6 to 9 days.

Table 3

Diffusion of $Na^{125}I$ through Thomas microfuge tubes at ambient temperature and 4°C.

	Ambient temp. % Total Counts in		4°C % Total Counts in	
Day	Buffer	Microtube	Buffer	Microtube
1	0,01	99,99	–	–
2	0,04	99,96	–	–
4	0,17	99,83	–	–
6	0,41	99,59	0,02	99,98
9	–	–	0,03	99,97

Total counts added 30 250 counts/second/0,25 mℓ.

After 6 days less than 0,5% of the $Na^{125}I$ had diffused out of the microfuge tubes at ambient temperature while at 4°C the loss was not significant for both 6 and 9 day incubations (Table 3). When the incubate contained radio-iodinated LH the loss was 0,2% after 6 days at ambient temperature, while no losses were recorded for up to 9 days incubation at 4°C (Table 4).

Table 4

Diffusion of radioiodinated LH through Thomas microfuge tubes at ambient temperature and 4°C.

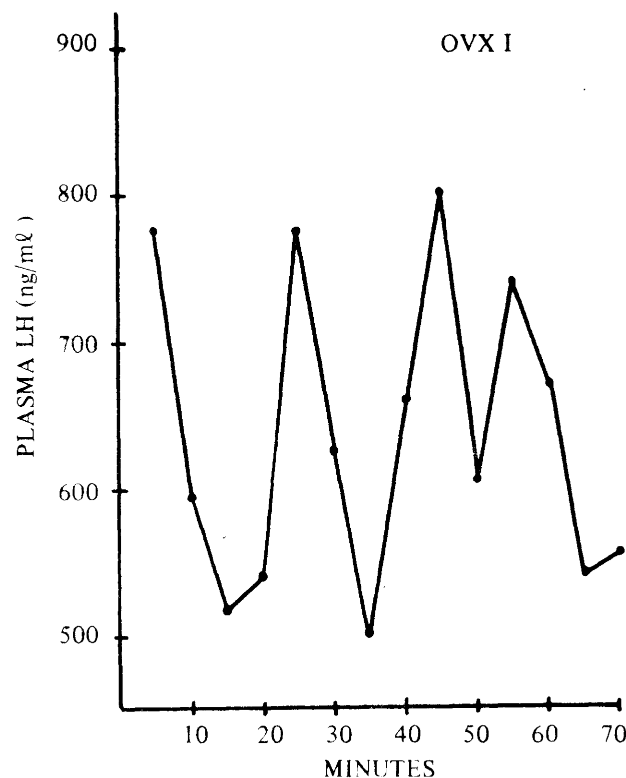
	Ambient temp. % Total Counts in		4°C % Total Counts in	
Day	Buffer	Microtube	Buffer	Microtube
1	0	100	–	–
2	0,07	99,93	–	–
4	0	100	–	–
6	0,20	99,80	0	100
9	–	–	0	100

Total counts added 19 320 counts/minute/0,25 mℓ.

Plasma LH concentrations

The mean plasma LH concentrations in normal, conscious, unrestrained male rats was 46,8 ± 27,2 ng/mℓ, while that in consicous dioestrous females was 77,9 ± 22,9 ng/mℓ. Three weeks after bilateral ovariectomy, the mean serum LH concentration rose to 701,7 ± 65,8 ng/mℓ, while measurements made on 42 sequentially collected blood samples from three ovariectomized rats showed that LH levels varied in a pulsatile manner (Fig. 3). The mean frequency of the pulses was one every 18,4 ± 4,5 minutes.

Fig. 3 *Pulsatile LH release in a conscious, unrestrained, chronically ovariectomized rat.*



Changes in serum LH levels during the normal oestrous cycle were measured in 3 female rats bearing indwelling jugular cannulae. Daily vaginal smears were examined and after each rat had completed 2 oestrous cycles, daily blood samples were aspirated at 16h00 for a further 2 consecutive cycles.

The mean dioestrous serum LH concentration was 78,2 ± 29,3 ng/mℓ. This value increased 4-fold to reach a pro-oestrous peak level of 345,3 ± 224,8 ng/mℓ ($P < 0,025$). At oestrus, the serum LH level had decreased to 74% of the pro-oestrous value (254,7 ± 79,8 ng/mℓ) but was still significantly higher than the dioestrous level, while at metoestrus, the hormone concentration had dropped to 77,5 ± 17,3 ng/mℓ (Fig. 4).

The effect of intravenous LRH on serum LH levels was measured in five chronically cannulated male rats. On separate occasions they were each given doses of LRH ranging between 50 and 800 ng LRH/0,2 mℓ, and blood samples were aspirated at various times up to 80 minutes thereafter. The log-dose response curve shown in Fig. 5 was derived by plotting peak plasma LH concentrations against the dose of LRH injected. The response was linear in the dose range employed.

Fig. 4 *Mean serum LH concentrations during the rat oestrous cycle. DI, PE, E. & M refer to dioestrous, pro-oestrous, oestrous and metoestrous stages of the cycle.*

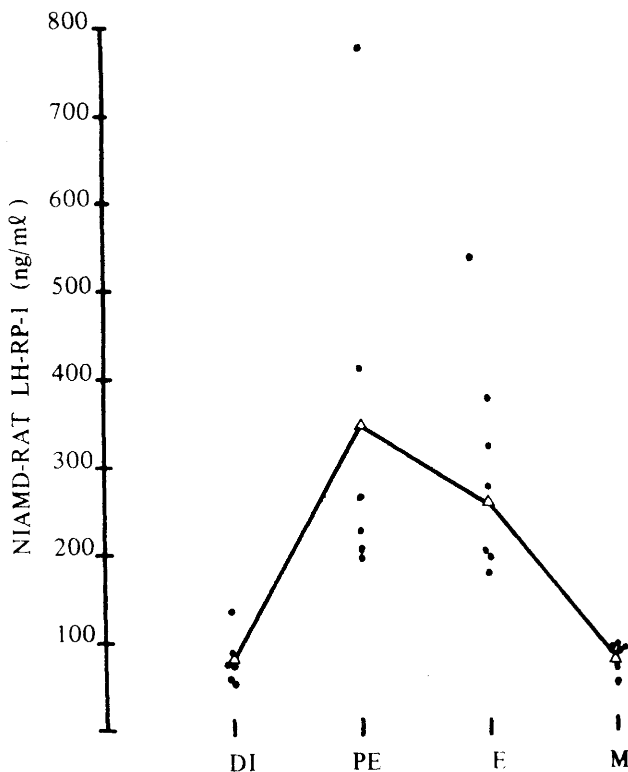
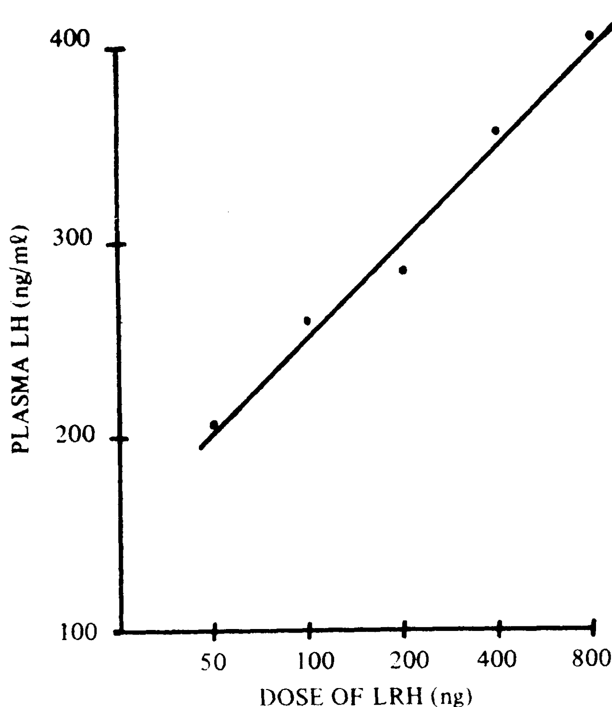


Fig. 5 *The peak plasma LH response to different doses of LRH showing a linear log-dose relationship.*



Discussion

The sensitivity of the assay (16 ng LH/mℓ) is in agreement with the work of Seki, Seki, Yoshihara & Maeda (1971) who reported a value of 15 ng LH/mℓ, using the same standard LH preparation. Furthermore, the coefficient of inter-assay variation (± 6,6%) compared favourably with the findings of Naftolin & Corker (1971) and Seki *et al* (1971) who reported values of ± 7,5% and ± 12,7% respectively. The coefficient of inter-assay variation using pooled serum specimens (± 6,4%) was also in close agreement with the findings of Naftolin & Corker (± 7,5%). The mean coefficient of intra-assay variation over the steep portion of the standard curve was found to be ± 3,1% as compared with ± 5% (using pooled serum specimens) reported by Naftolin & Corker (1971).

In order to elimate errors associated with inter-assay variability, all unknown samples from a particular experiment were routinely included in a single assay. Furthermore the results of independent studies were compared only when the inter-assay variation of the internal pooled serum standard fell within the limits defined above. In order to improve further the reliability of the LH measurements, all samples expected to show LH levels greater than 250 ng/mℓ were serially diluted. Not only did this procedure provide a check on the parallelism of the assay, but it also gave a reading off the steeper and hence the more sensitive region of the standard curve.

The demonstration of parallelism between serum LH and standard LH has added further weight to the validity of the assay procedure, by indicating comparable immunological behaviour of unknown and standard solutions.

Evidence for leakage of radioactive iodide from the assay microtubes was provided by Kuhn & Naftolin (1975) who showed this to be very marked from tubes incubated at room temperature for 2 to 3 days. They also demonstrated that radio-iodinated FSH was lost in significant amounts after the same periods of incubation. However, in the present study, their findings could not be repeated even though the dose of radio-iodine used was 100 times greater than that employed by these workers. The microfuge tubes used in the two investigations are probably not identical. Those used in the present investigation were made in Switzerland and supplied by Thomas Scientific Company, while the tubes employed in the Kuhn & Naftolin study were supplied by Beckman Instrument Company. The possibility that different batches of tubes behave differently, however, cannot be overlooked. It would thus be advisable to test new batches of tubes for leakage of this sort.

The females of the strain of rat used in this study tend to remain in constant oestrus and are thus relatively infertile (e.g. of ten monogamously mated pairs only 4 females became pregnant after 2 weeks). The hormonal changes in these rats during the oestrous cycle, although resembling those found by other workers (Naftolin, Brown-Grant & Corker, 1972; Ramirez & McCann,

1964) show a relatively low pro-oestrous peak when measured at 16h00. Recent observations have shown, however, that these animals exhibit a pro-oestrous LH rise which only begins at 16h00 and peaks at 20h00 or later (Beardwood & Kellaway, unpublished observation).

Removal of the ovaries caused the plasma LH concentration to rise 8-fold, an effect observed by other workers (Parlow, 1964; McCann & Ramirez, 1964). In addition the pulsatile nature of LH secretion could be clearly demonstrated. The average frequency of these pulses was one every 18,4 ± 4,5 minutes which is comparable with the rate of one in 24 minutes derived from the recent work of Blake & Sawyer (1974).

In addition to the data obtained in female rats, the dose response curve for LRH stimulation of LH release in male rats is in agreement with the earlier report of Gay, Niswender & Midgley (1970). A dose of 50 ng LRH per rat induced a statistically significant rise in LH output and this response was doubled at a dose of 800 ng LRH per rat.

It is thus clear that the reliability criteria for this assay are in good agreement with earlier reports employing similar reagents. Furthermore, the ability of this technique to record faithfully the well documented changes in gonadotrophin levels which occur in different physiological states confirms its specificity as an immunoassay for rat serum LH measurement.

Acknowledgements

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