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EQUINE LUTEINIZING HORMONE

MEASUREMENT AND REGULATION

OF SERUM LEVELS

A THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

UNIVERSITY OF CANTERBURY

by

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Lincoln College

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The experiments described in this thesis were carried out by myself except where assistance was given as indicated in the Acknowledgements.

The results of experiments in Section 1, Chapter 2, will be published in Supplement 32 to the Journal of Reproduction and Fertility, as will results of experiments in Section 4.
I would like to express my sincere appreciation to a number of people without whose help this project would have been all the more difficult to complete:

For performing field work, I thank Miss Janet Little, Miss Leonne Gason, Dr. Margaret Evans, Dr. Robert Loy, Prof. C.H.G. Irvine and most especially Miss Julie Turner, who also donated geldings, Poldark, Gumboots and Junior, for the experiments described under Section 4. I would also like to express my gratitude to Nevele R Stud, Prebbleton, for use of horses and facilities, and for the unfailing cooperation of studmaster, Michael Butler, and his staff. The willingness of veterinarians, Bruce Taylor, Bill Bishop, John Shaw, Corin Murfitt and Basil Forsyth, in supplying endless pairs of horse testicles for attempts at equine _in vitro_ bioassays is most appreciated.

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In preparing this manuscript, I must thank all my typists, Kathy Brown, Julie Lassen, Anne Lawson and C.H.G. Irvine, who performed so valiantly under pressure. I am also very grateful to Margaret Evans for applying her artistic talents to the text figures and to Mr. Kim Prisk and his computer for figures in Section 3.
Finally, I would like to express my gratitude to my supervisors, Dr. Margaret Evans and Prof. C.H.G. Irvine for reading and critically evaluating this thesis. I am particularly grateful to Prof. Irvine for providing the funds, facilities, and, most importantly, the inspiration for these studies.
An in vitro bioassay based on LH-stimulated testosterone production by dispersed mouse Leydig cells was validated for use in the horse. Using this bioassay and a previously validated heterologous radioimmunoassay (RIA), it was found that the patterns of LH levels measured by the 2 assay methods during the mare's oestrous cycle were similar but not identical, so that the ratio of biological:immunological (B:I) activity changed significantly during the cycle; being high as LH levels rose during oestrus but falling sharply on the last day of oestrus to remain stable and low through dioestrus.

Investigating factors affecting the B:I ratio of serum LH, it was observed that the low ratio in serum from seasonally acyclic mares could be raised by pre-treatment with oestradiol, followed by pulse injection of gonadotrophin-releasing hormone (GnRH). Neither GnRH nor oestradiol treatment alone could alter B:I ratio in acyclic mare serum. Thus, it was suggested on the basis of this observation and review of relevant literature that the rising serum oestradiol levels preceding ovulation and increased GnRH levels which may occur at this time could contribute to the presence in serum of LH forms with greater biological potency. Changes in B:I ratio during the oestrous cycle implied qualitative differences in the circulating molecule. The nature and source of these differences were investigated by using the technique of isoelectric focussing (IEF) to separate, on the basis of charge,
the various forms of LH in pituitary extracts and serum samples. Isoelectric focussing of pituitary extracts resulted in LH peaks at pH 7.2, 6.1, 5.2 and 4.5, with differing B:I ratio; mean ratio being greatest in the pI 5.2 peak and least in the 7.2 peak. When horse serum was focussed, marked similarities between serum and pituitary LH were observed with peaks of LH activity occurring at like pI values and B:I ratio changing similarly with the pI of peaks. Serum LH was different to pituitary LH in that immunoactivity without bioactivity was found at either pH extreme. When IEF profiles of high and low B:I ratio sera (paired samples from 2 cyclic mares) were compared, no consistent shift in LH distribution was observed with decreased ratio; however, in both low ratio sera, relatively more LH activity was found in areas of low B:I ratio and consequently less LH activity was recovered in the highest ratio peak. While interpretation of these results was complicated by the small number of samples focussed, it appeared that changes in the B:I ratio of serum LH could be related to structural changes in the circulating molecule. Whether the polymorphism of serum LH originated entirely from the pituitary or in part from post-secretory modification of the molecule could not be conclusively answered. The close correspondence between pI values at which the bulk of pituitary and serum LH focussed attested to the pituitary being the major source of this polymorphism; however the presence in serum but not pituitary of LH with little or no bioactivity suggested that some post-secretory mutation might occur. This question was raised again in Section 3, when little correlation could be found between patterns of bio- and immuno-active LH levels in serum samples collected at 5 or 15-20 min intervals from cyclic mares. In oestrous mares, rapid, low amplitude pulses in serum LH
levels were shown to occur with statistically significant regularity when measured by bioassay but not by radioimmunoassay. Results with dioestrous mares were highly variable with pulses similar in amplitude and frequency to those observed at oestrus demonstrable in some, but not all, mares. In dioestrous mares in which LH pulses could not be shown and in seasonally acyclic mares, serum LH levels remained stable and did not appear to decay during the period of observation regardless of assay method.

The physiological significance of the above observations requires further investigation; nevertheless, the fact that the mare ovulates when relative biopotency of serum LH is maximal as assessed by mouse Leydig cells, suggests both that qualitative changes in the circulating molecule do have physiological importance and that the response of equine target cells can be predicted adequately by the mouse (a supposition supported by preliminary results from an in vitro bioassay using horse Leydig cells).

The practical significance of these findings is that there appear to be circumstances in which the radioimmunoassay used here would not give adequate information on the level of LH stimulation at target tissues. Furthermore, it is evident that bio- and immunoassay measure forms of the hormone which, especially at oestrus, appear in serum with different time courses.

In the course of experiments in which GnRH was given to mares to determine the effect of stimulation on the nature of the circulating LH molecule it was observed that both bio- and immuno-active LH responses to a small dose of GnRH (i.e. "pituitary responsiveness") were lower in early oestrus than in early dioestrus, whereas pre-injection LH levels were higher in oestrus than dioestrus. Furthermore, additional experiments demonstrated that in the mare the ovulatory LH
surge began without a corresponding increase in pituitary responsiveness to GnRH stimulation suggesting that increased GnRH input to the pituitary was responsible for the onset of the LH surge. The basis for this deduction was proved to be less than firm when subsequent experiments failed to demonstrate a GnRH dose-LH response relationship in oestrous mares, with GnRH doses ranging from 0.05 mg to 2.0 mg eliciting similar responses. In the absence of a GnRH dose-LH response relationship, estimates of pituitary responsiveness (i.e. LH release/unit GnRH) would vary with GnRH dose given, and therefore the response of the pituitary to endogenous GnRH input could not be inferred from response to exogenous GnRH administration. Thus, a GnRH dose-LH response relationship must be shown in oestrous mares before the relative importance of changes in pituitary responsiveness and endogenous GnRH secretion in producing the ovulatory LH surge can be confidently assessed.

In a series of experiments, the regulation of seasonal patterns of LH secretion in the male horse was studied. In particular, the importance of the testes in maintaining the annual pattern of serum LH levels was investigated by measuring LH levels in blood samples collected at approximately fortnightly intervals for a year from 5 "long-term" geldings (castrated > 3 years). In these horses, no significant effect of month on LH levels was observed. By contrast, earlier work in our laboratory had shown that stallions in the same environment during one year have a markedly seasonal pattern of LH secretion, with LH levels rising at the onset of the breeding season to reach levels in late spring 3-4 times those in early winter. Despite differences in seasonal patterns of secretion, annual mean LH levels in geldings and stallions were similar. In mid-summer, serum LH levels were measured in 5
geldings castrated 4-6 weeks previously, 24 geldings castrated 3-25 years previously and 5 stallions; mean LH levels ± S.E.M. (in ng/ml) were: 63.5 ± 8.8, 14.0 ± 1.6 and 9.0 ± 1.5 respectively. In the long-term geldings LH levels were not affected by age, indicating that following the initial post-castration rise, LH levels fell to within the normal range of pre-castration values and stabilized.

These results show that the testes are necessary for maintenance of the normal seasonal pattern of LH secretion in the male horse, including the increase in LH at the onset of the breeding season. Thus, it is possible that the effects of testicular hormones on LH secretion in the male horse may include not only a negative feedback at the hypothalamus/pituitary as observed in other species but also a positive component in the pathway by which LH is increased at the onset of the breeding season.
SECTION 1.

EQUINE LH: STRUCTURE-FUNCTION RELATIONSHIPS
INTRODUCTION

Equine pituitary luteinizing hormone (eLH) is a glycoprotein with a molecular weight of 34,000 (Braselton and McShan, 1970). Like all luteinizing hormones studied, eLH consists of two dissimilar subunits (alpha and beta) held together by noncovalent bonds (Landefeld and McShan, 1974). Within species, the alpha subunits of LH, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) have been found to be essentially identical, whereas the beta subunit seems to possess the biological specificity of the hormone (Pierce and Parsons, 1981). The carbohydrate content of eLH is 23.6% by weight (Landefeld and McShan, 1974) which is higher than that reported for any other luteinizing hormone (see Table 1). Equine LH is also unusual in that it is heavily sialylated (sialic acid content = 7.7% by weight, Landefeld and McShan, 1974). Of other luteinizing hormones, only human LH has been shown to contain appreciable amounts of sialic acids (Kathan, Reichert and Ryan, 1967). When eLH is subjected to isoelectric focussing, it is found to be markedly polymorphic, consisting of a family of molecules of varying charge (Braselton and McShan, 1970; Reichert, 1971; Irvine, 1979). These differences in charge seem to arise in part from variations in content of the strongly negative sialic acid (Irvine, 1979; Aggarwal and Papkoff, 1981). Human LH is also polymorphic, as is clearly revealed by isoelectric-focussing (Reichert, 1971; Robertson, van Damme and Diczfulusy, 1977), but this polymorphism disappears after neuraminidase digestion, suggesting that it stems from variable sialylation (Reichert, 1971). Recent work shows that enzymatic removal of sialic acid from equine and human gonadotrophins differentially affects potency in various biological assays and radioimmunoassay (see Discussion, this section, for review of literature). More interestingly, the various forms of equine
pituitary LH separated by isoelectric focussing have been observed to differ in relative activities in radioimmunoassay and in vitro bioassay (Irvine, 1979). Because the nature of serum equine LH is unknown, the validity of radioimmunoassay for measurement of biologically active hormone could be questioned especially if, as in the human (Strollo et al., 1981), rhesus monkey (Peckham, Yamaji, Dierschke and Knobil, 1973), and rat (Weick, 1977) the steroid environment can alter the form of the circulating hormone. The experiments in this section were therefore designed: 1) to investigate the relationship between biological and immunological LH activities in serum throughout the oestrous cycle of the mare; 2) to determine the effect of gonadal steroids and gonadotrophin releasing hormone (GnRH) alone and in combination, on relative biological and immunological LH activities in serum, and 3) to compare isoelectric focussing profiles of pituitary and serum LH.
Table 1: Carbohydrate composition of LH, hCG and PMSG.

<table>
<thead>
<tr>
<th>Hormone + Species</th>
<th>Carbohydrate %</th>
<th>Sialic Acid (residues/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ovine</td>
<td>13.0</td>
<td>0</td>
</tr>
<tr>
<td>bovine</td>
<td>12.2</td>
<td>0</td>
</tr>
<tr>
<td>porcine</td>
<td>13.2</td>
<td>0</td>
</tr>
<tr>
<td>equine</td>
<td>23.6</td>
<td>8.5</td>
</tr>
<tr>
<td>human</td>
<td>16.4</td>
<td>2.3</td>
</tr>
<tr>
<td>hCG</td>
<td>29.0-30.3</td>
<td>9.5-10.9</td>
</tr>
<tr>
<td>PMSG</td>
<td>44.4</td>
<td>9.4</td>
</tr>
</tbody>
</table>

1 Adapted from Sherwood and McShan, 1977
CHAPTER I.

ASSAY METHODOLOGY
The assay chosen to measure "biologically active" LH was an in vitro testosterone production assay using mouse interstitial cells (van Damme, Robertson and Diczfalusy, 1974b). In this assay, interstitial cells, dispersed from mouse testes, are stimulated during a 3-hour incubation to secrete testosterone in response to LH in standards and unknowns. Secreted testosterone is then measured by radioimmunoassay (see Figure 1). This assay had several major advantages over other potential choices: 1) it was highly sensitive and could easily detect LH levels in dioestrous and seasonally acyclic mares (unlike in vivo bioassays and radioreceptor assays); 2) mouse interstitial cells could be separated from seminiferous tubules without collagenase digestion, thereby reducing the risk of receptor damage (see Reichert and Abou-Issa, 1976, re rat FSH receptor), and altered cell responsiveness (collagenase digestion is required to harvest cells from rat testes); 3) mice were simply and inexpensively kept, meaning that cells for use in the assay were always available (unlike in vitro bioassays using bovine corpus luteum or pig granulosa cells); 4) it measured LH by its ability to perform an accepted physiological function (unlike the Leydig cell alkaline phosphatase induction assay (Ryle and Carrier, 1981) or cytochemical method based on ovarian ascorbic acid depletion (Buckingham et al., 1979)); and 5) using this assay, fractions after isoelectric focusing of horse pituitaries has been found to vary in relative "biological" and radioimmunological potencies (Irvine, 1979).

The assay chosen to measure "immunologically active" LH was a heterologous system consisting of anti-ovine LH, labelled ovine LH and equine standards. This assay was selected because of its widespread use to measure equine LH (see, for example: Pattison, Chen, Kelly and Brandt,
Mouse Leydig cells + LH $\rightarrow$ testosterone

Mouse castrated.
Testes separated into tubules and interstitial cells by gentle stirring.

Incubate 3 h at $34^\circ$C.

Measured by radio-immunoassay.
I. IN VITRO BIOASSAY

Animals: Mice were NZ white x NZ black male hybrids (gift from Christchurch Clinical School). They were kept in groups of 2 - 7 in 19.5 x 32 cm plexiglass cages (Christchurch Clinical School) at ambient laboratory temperature (13 - 18°C) under natural photoperiod. Pelleted mouse ration (No. 95, H. Archer & Son Ltd, Southbrook) and water were fed ad libitum.

Preparation of Cells: Adult mice (> 2½ months) were killed by cervical dislocation, the testes removed, decapsulated and placed in a 25 ml glass Erlenmeyer flask containing 10 ml tissue culture medium (TCM, see later for preparation). The testes were gently teased apart by a glass rod and further dissociated by slow stirring on a magnetic stirrer for 15 min at room temperature, after which seminiferous tubules were separated from interstitial cells by filtration through 4 layers of gauze bandage. The cells were then incubated in TCM for 1.5 - 2 h at 34°C in a shaking water bath set at 80 - 100 r.p.m. After incubation, the cells were washed with 10 ml fresh TCM at 4°C 3 - 4 times, each wash being followed by centrifugation at 600 x g for 5 min at room temperature. After the last wash, cells were resuspended once again in 10 ml cold TCM and counted in a haemacytometer (Neubauer improved). On some occasions, viability was also assessed by exclusion of Trypan blue dye (0.06% trypan blue, in 0.154 M sodium chloride, 0.01 M phosphate buffer, pH 7.4). In general, cell types were not classified (van Damme et al., 1974); however, red blood cells and spermatozoa when present were not counted. Seminiferous tubules were seldom seen in any preparation. The mean number of interstitial cells harvested from a pair of testes was 12.0 x 10^6 (S.D. = 6.2 x...
$10^6$, $n = 28$ randomly selected assays) 65 - 75% of which excluded trypan blue.

In the original method, the tissue culture medium used was Medium 199 with Hank's salts and l-glutamine (Catalogue No. E-12, GIBCO Grand Island, N.Y., U.S.A.) with addition of 1.4 g/l of sodium bicarbonate and 0.5 - 1.0% fetal calf serum (Batch 107, Catalogue No. 614, GIBCO, heated to 56°C for 0.5 h and stored at -20°C in 500 µl aliquots). The pH was adjusted to 7.3 - 7.4 with sodium bicarbonate or 1 N hydrochloric acid. No attempt was made to maintain sterile conditions; the TCM was not filtered and antibiotics were not added. Incubation of cells was performed in a specially constructed cannister designed to fit the trolley of the shaking water bath and in which a humidified atmosphere of 5% CO₂:95% O₂ could be maintained (see Figure 2).

In the modified method, the medium used was Eagle's minimum essential medium with Earle's salts* and l-glutamine (Catalogue No. F-11, GIBCO) containing 0.5 - 1.0% fetal calf serum and buffered with 35 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, No. H-3375, Sigma Chemicals Co., St Louis, Missouri, U.S.A.) as described by Lichtenberg and Pahnke (1976). The pH was adjusted to 7.3 - 7.4 with 1 N sodium hydroxide or 1 N hydrochloric acid.

Changing the buffer allowed incubation to be performed under air thereby reducing the cost of the assay, increasing the number of samples that could be assayed together and reducing variability in incubation conditions as assessed by colour change during incubation of the pH indicator, phenol red, in the TCM. Assay results were not affected by

*The author is well aware of the misuse of Hank's and Earle's balanced buffer salts. The method for preparing Medium 199 was inherited (as was the M199) from another student and when the error in usage of Hank's salts was eventually discovered, tissue culture medium with the appropriate Earle's salts was ordered. However, shortly thereafter, it was decided to try using HEPES to buffer the medium - hence HEPES buffer and Earle's salts.
Figure 2. Cannister used for incubating the in vitro bioassay under 5% CO$_2$ in O$_2$. 

![Cannister image](image-url)
changing the buffer. Mean LH concentrations \( \pm \) SEM measured in a replicate serum standard by the last two* bicarbonate assays and first five HEPES assays were 19.2 \( \pm \) 1.1 ng/ml and 18.2 \( \pm \) 0.6 ng/ml respectively. Furthermore, when daily serum samples from two cyclic mares were assayed by both methods, the mean ratio \( \frac{\text{potency estimate HEPES}}{\text{potency estimate bicarb}} \pm \text{SEM} \) was 1.03 \( \pm \) 0.07, \( n = 26 \) samples, each potency estimate being the mean of triplicate determinations. The mean slope \( \left( \frac{\Delta \text{logit} B/BO}{\Delta \log LH} \right) \), see later for calculation) of standard curves in the last 4 bicarbonate assays was 1.53 (range = 1.14:2.05) compared with 2.49 (range = 1.95:3.06) in the first 4 HEPES assays; a significant improvement (\( p < 0.05 \), Student's t-test). Neverthe less, 17 two-point dilutions of serum in the last 4 bicarbonate assays were parallel to the appropriate standard curve and 11 two-point serum dilutions in the 4 HEPES assays were parallel to the HEPES standard curves. The method for determining parallelism will be discussed later.

Lichtenberg and Pahnke (1976) reported that mouse Leydig cells could be kept in TCM overnight at \( 4^\circ \) with no loss of viability. In the present studies, it was found that the period of successful storage extended to at least 3 days after cell harvest when dispersed cells from 2 mouse testes were stored at \( 4^\circ \) in 10 ml TCM prepared as described previously. Successful storage is illustrated by the results of the experiment shown in Figure 3. Furthermore, when serum replicate standards were assayed on consecutive days using cells one and two days old, potency estimates were not significantly affected by age of cell (paired t-test, \( t = 0.04, \ n = 8 \) paired experiments using two different serum pools).

Storage of cells increased the efficiency with which the assay could be performed, since cells could be prepared once at the beginning of a week and used as required throughout the week.

* New replicate standards were introduced at this time.
Figure 3: Effects of age of cell on the in vitro bioassay

Protocol

Day 1: LH standards were prepared. Each standard was divided into 4 aliquots and frozen.

Day 2: a) One mouse was castrated and an interstitial cell suspension was prepared.
   b) Cell viability was assessed by trypan blue exclusion.
   c) One set of LH standards was thawed, aliquotted into Wasserman tubes and cells added.
   d) Incubation proceeded as described earlier. Remaining cell preparation was stored at 4°C. Incubation medium was stored frozen until testosterone assay.

Days 3-5: Steps b) - d) were repeated, after an appropriate aliquot of stored cell preparation had been washed once.

Results

<table>
<thead>
<tr>
<th>Approximate number of cells added to each tube</th>
<th>Age of Cell</th>
<th>(In Days After Harvest)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>75,000</td>
<td>60,000</td>
<td></td>
</tr>
<tr>
<td>% live cells</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pg LH</th>
<th>fg testosterone*</th>
<th>per cell *</th>
<th>(mean of triplicate observations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>8.2</td>
<td>6.9</td>
<td>9.6</td>
</tr>
<tr>
<td>50</td>
<td>5.7</td>
<td>3.9</td>
<td>7.3</td>
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<td>25</td>
<td>2.7</td>
<td>2.1</td>
<td>4.1</td>
</tr>
<tr>
<td>12</td>
<td>0.88</td>
<td>0.65</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>0.30</td>
<td>0.52</td>
</tr>
<tr>
<td>0</td>
<td>0.14</td>
<td>0.12</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*Corrected to take into account differences in cell viability.

Means without a common superscript are significantly different at the 0.05 level (analysis of variance, Tukey's W test; Steel and Torrie, 1980).

Conclusions

Although ANOVA showed that "cell age" significantly affected testosterone production, there was no evidence for a progressive deterioration in cell performance. In fact, mean testosterone production/cell was greatest on the second day after harvest. Because of increasingly noticeable bacterial contamination and decreasing cell viability, cells older than 2 days were not used for routine assays.
The modified assay procedure was used after 15/1/80, but since potency estimates did not appear to have been affected by the change in methodology, date of assay has not been noted in results.

The Assay: Standards and unknowns were diluted with "assay diluent" (Robertson and Diczfalusy, 1977), which consisted of 0.15 M sodium chloride (NaCl), 0.1% bovine serum albumin (No. l-4503, Sigma Chemical Co.) in distilled water. The pH of this diluent was generally 5.95 - 6.05. Dilutions were made in and the assay performed in 12 x 75 mm disposable polystyrene test tubes ("Wasserman tubes", Laboratory Services, Penrose, N.Z.).

The standard used was equine pituitary LH, prepared by Prof. C.H.G. Irvine at Texas A & M University, using the following method. Pooled horse pituitaries were extracted with ethanol, precipitated with metaphosphoric acid (Braselton and McShan, 1970) and the resulting supernatant fractionated by isoelectric focussing. The fractions with the highest ratio of in vitro biological:immunological LH activity (Irvine, 1979) were retained for use as assay standard and were designated CI 1-37. CI 1-37 contained 84.8 μg LH (by in vitro bioassay, expressed in terms of bovine LH, LER 1072-2, 1 ng = 1.8 ng NIH LH-S1), 521 μg FSH (by radioimmunoassay, expressed in terms of Nuti equine FSH, 1 ng = 90 ng NIH FSH S-1), and 17.3 mg protein as measured by the method of Lowry et al. (1960). After these initial assays, CI 1-37 was lyophilised and reconstituted in 1 ml 0.154 M NaCl, 1% bovine serum albumin in distilled water. Because of the impurity of the preparation, it was most convenient to define its potency in terms of DILUTIONS producing in vitro biological LH activity equivalent to known amounts of LER 1072-2. For example, 100 ul of a 1 in 20,500 dilution of CI 1-37 was found to have LH activity equal to 410pg LER 1072-2, and therefore this dilution was said to produce 410pg CI 1-37/100 ul.
The decision to use CI 1-37 as standard was based on several considerations: 1) Equine pituitary is polymorphic. The crude pituitary extract from which CI 1-37 was produced would have been more likely to contain, unaltered, the full spectrum of LH molecules in the pituitary than would highly purified laboratory preparations produced by rigorous, sometimes harsh, separation techniques. 2) The ideal standard should be identical to the substance measured. In most experiments, biologically active LH in serum was the substance to be measured and it was assumed that this would be most closely similar to the molecular form of pituitary LH with the greatest ratio of biological:immunological activity. Further purification of CI 1-37 was decided against because of the risk of damage to the molecule and because the high specificity of the assay made such steps unnecessary (see later).

The standard was stored at -20\(^\circ\) in 50 \(\mu\)l aliquots containing 8.4 ng CI 1-37 in 0.154 M NaCl, 1% bovine serum albumin. Before each assay, an aliquot was thawed and 2 ml assay diluent added to produce the top standard containing 410 pg CI 1-37/100 \(\mu\)l. Serial 1:1 dilutions were then made with assay diluent giving the following concentrations of standards: 205 pg/100 \(\mu\)l, 102.5 pg/100 \(\mu\)l, 51.3 pg/100 \(\mu\)l, 25.6 pg/100 \(\mu\)l, 12.8 pg/100 \(\mu\)l, 6.4 pg/100 \(\mu\)l and 32 pg/100 \(\mu\)l. Serum samples were also diluted in assay diluent. The amount of serum used in the assay was generally 0.5 - 2 \(\mu\)l; however, 4 - 8 \(\mu\)l of an oestrous mare serum and 0.125 - 0.25 \(\mu\)l of serum from mares in oestrus or after gonadotrophin-releasing hormone (GnRH) administration were sometimes needed.

For the assay, the cell preparation was diluted with cold TCM to a concentration of 30,000 - 60,000 cells/100 \(\mu\)l. A homogeneous suspension was ensured by gentle mixing on a magnetic stirrer. One hundred \(\mu\)l of the cell preparation was then added to 100 \(\mu\)l diluted unknown or standard in Wasserman tubes. These steps were performed at 4\(^\circ\). Assay tubes were vortexed at slow speed, placed in a shaking water bath and incubated at 34\(^\circ\).
for 2.5 - 3 h. When the TCM was bicarbonate buffered, this incubation occurred in the special cannister under 5% CO₂ - 95% O₂, whereas when HEPES was the buffer, air was the gas phase. After incubation, assay tubes were kept at 4°C while the temperature of the water bath was brought to 72 - 75°C, and were then returned to the bath for 0.5 h. In both bicarbonate and HEPES systems, heating was done under air. Temperatures greater than 80°C were found to melt the Wasserman tubes. This heating step was reported to be necessary to destroy a testosterone binding substance in the interstitial cell preparation (van Damme et al., 1974a). Condensation was removed from the walls of the assay tubes by centrifugation at 1000 x g/2 min, after which the testosterone content of the medium was determined by radioimmunoassay (RIA).

Testosterone Radioimmunoassay: The anti-testosterone serum used was GDN S-250 (antigen = testosterone conjugated to BSA at position 11, gift from Dr G.D. Niswender, Colorado State University, Fort Collins, Colorado, U.S.A.) which cross-reacted 100% with testosterone, 33% with di-hydrotestosterone, 10% with 5α-androstan-3α-17β-diol and 5% with 5α-androstan-3β-diol (de Palatis et al., 1978). The antiserum was diluted 1 in 400 with "testosterone assay buffer", which consisted of 0.01 M phosphate (PO₄) buffer (i.e., 0.295 g/l NaH₂PO₄, 1.15 g/l Na₂HPO₄), 0.145 M NaCl, 0.025 M ethylene di-amine tetra acetic acid-disodium salt (EDTA), 0.02% sodium azide (NaN₃) and 0.1% gelatin (Davis, Christchurch), pH 7.0. One ml aliquots were stored at -20°C. Before use, the antiserum was further diluted to 1 in 10,000 in assay buffer.

An aliquot of tritium-labelled testosterone (1,2,6,7,³H-testosterone, specific activity 80 Ci/mmol, Radiochemical Centre, Amersham, England) was suspended in assay buffer to an approximate concentration of 370 pg/ml (or 100,000 cpm/ml at 45% counting efficiency). This solution was combined 2 parts:1 part with the 1 in 10,000 dilution of antiserum, and 300 µl of this mixture was added directly to all assay tubes (i.e.,
tubes containing cell preparation and LH standards or unknowns).

Because testosterone is the major product of mouse Leydig cells, solvent extraction and/or chromatography was not necessary (van Damme et al., 1973). Also included in the testosterone assay were tubes containing:

1) 100 μl tissue culture medium, 100 μl assay diluent and 300 μl \(^3\)H-testosterone/antiserum mixture (= zero added testosterone); 2) 100 μl cell preparation, 100 μl assay diluent, 200 μl \(^3\)H-testosterone solution and 100 μl assay buffer (= "blanks", to determine the amount of tracer in the charcoal supernatant not bound to antibody); 3) 200 μl \(^3\)H-testosterone solution and 300 μl assay buffer (= total radioactivity added to the system); and occasionally 4) testosterone standards.

(Calculation of assay results did not always involve quantitating the amount of testosterone produced by cells. See later for details of assay analysis.) These standards were made by dissolving testosterone (Δ4-androstan-17β-ol 3-one, Sigma Chemicals) in ethanol to a concentration of 1 μg/ml. One ml of this was evaporated under air and the testosterone redissolved in 10 ml assay buffer. This 100 ng/ml testosterone stock solution was stored at -20°C in 200 μl aliquots. Before use in the assay, 800 μl of TCM was added, resulting in the top standard containing 2 ng testosterone/100 μl. Serial 1:1 dilutions were then made with TCM to produce the following concentrations: 1 ng/100 μl, 0.5 ng/100 μl, 0.25 ng/100 μl, 0.125 ng/100 μl and 0.0625 ng/100 μl; 100 μl of each was pipetted into Wasserman tubes containing 100 μl assay diluent.

The assay was incubated at 37°C for 1 h, then at 4°C overnight.

Free testosterone was separated from antibody bound by charcoal adsorption; the charcoal suspension consisting of 0.4167% activated charcoal (BDH Laboratory Chemicals, Poole, England), 0.1 M NaH\(_2\)PO\(_4\), 0.15 M NaCl, 0.05% NaN\(_3\) in distilled water, pH 7.3. Originally, the charcoal suspension also contained 0.025% dextran; however, soon after the start of these experiments, this chemical became impossible to obtain. Tests comparing
dextran-coated and dextran-less charcoals showed no difference between the two in counts specifically or non-specifically bound after either 5 or 12 minutes incubation at 4°C, and therefore dextran-less charcoal could be used. Five hundred μl of charcoal suspension was added at 4°C to batches of approximately 65 tubes (the number of tubes that could be "charcoaled" in less than 5 min). The tubes were vortexed and allowed to stand at 4°C for 10 - 12 min, after which they were spun at 100 x g for 10 min in a refrigerated centrifuge (0°C - 4°C). Supernatants were decanted into glass scintillation vials, and 8 ml toluene scintillant (4 g diphenyl-oxazole, 0.2 g dimethyl POPOP in 1 l toluene (industrial grade, filtered through Whatman No. 541 hardened ashless filter paper before use) added. Vials were capped, vortexed for 7 - 10 secs to extract testosterone into the scintillant and counted in standard liquid scintillation counting equipment, usually until at least 10,000 counts/vial had accumulated. Samples were assumed to be counted with identical efficiency; however, this assumption was tested occasionally using the "external standard channels ratio" method (Long, E.C., 1976). In both counters used in these studies, the external standard channels ratio exceeded 0.7 for all samples in tested assays. Variations in ratio appeared to be randomly distributed; maximum deviation from the mean being 7%. Therefore, the assumption of identical counting efficiency for samples was considered valid.

**Assay Design:** Assay design changed during the course of this study, and was adapted to the requirements of each experiment*. The basic design was one-point assays of unknowns in triplicate, with 15 - 20% of unknowns also assayed at a 1:1 dilution. In most experiments in which GnRH was given, all samples were assayed at one dilution, then pre- and post-injection samples were separately pooled and the pools assayed at two

*And when relevant will be noted with descriptions of experimental method.
dilutions \( d_{\text{dilution one}} = 2 \). Most recently and particularly in experiments studying the short-term fluctuations in serum LH, the assay has been partially and finally completely randomised. In "partial" randomisation, the order in which samples occurred in the assay was random, but triplicates were kept together. In complete randomisation, tubes were ordered according to a table of random numbers before addition of cells and were returned to numerical order when the charcoal supernatant was decanted into scintillation vials.

**Assay Analysis:** The testosterone assay was analysed by transforming counts antibody bound to logit \( B/Bo \): logit being \( \log \frac{B/Bo}{1-B/Bo} \) where \( B = \) counts antibody bound in the presence of added testosterone and \( Bo = \) counts bound in the absence of added testosterone. When logit \( B/Bo \) was plotted against log testosterone added, a straight line \( (r \approx 0.995, \text{ least squares linear regression}) \) always resulted. The presence of non-antibody bound counts in charcoal supernatants was corrected for as described by Harris (1980). Briefly, it was assumed that in any tube the ratio of counts charcoal bound:counts free (i.e., neither charcoal nor antibody bound) would be a constant which could be determined using "blank" tubes (i.e., tubes containing all reagents except antibody). For example, if total counts added to the system = 10,000, and counts in the charcoal supernatant of the "blank" = 200, then counts charcoal bound = 9800 and counts free = 200. Thus, the ratio charcoal bound:free is 49 (i.e., \( \frac{9800}{200} = 49 \)). Extending the example to an assay tube containing antibody in which counts in the charcoal supernatant = 2000 (i.e., counts antibody bound + counts free); here, counts charcoal bound = 8000. If \( \frac{\text{counts charcoal bound}}{\text{counts free}} = k = 49 \), then free = \( \frac{\text{counts charcoal bound}}{49} \) or \( \frac{8000}{49} = 163 \), and counts antibody bound = \( 2000 - 163 = 1837 \).
No completely satisfactory way of linearising the LH dose-testosterone response curve has been described. Partial linearisation could be achieved by plotting: 1) logarithm of the LH dose given against the square root of the amount of testosterone produced (van Damme et al., 1974); 2) LH dose given against stimulated testosterone production (testosterone produced at given dose - testosterone produced in the absence of added LH) (Alexander and Irvine, 1980); or 3) logarithm of LH dose against logit \( B/Bo \) where \( B = \) counts bound to the testosterone antibody in standard/sample tubes, and \( Bo = \) counts bound to the antibody in the absence of added LH (Robertson, 1977). Occasionally, \( Bo \) was defined as counts bound in the absence of added testosterone, if a better LH dose-response curve resulted (Robertson, pers. comm.). Of these methods, 3) was preferred because of its simplicity (calculation of testosterone produced was not required) and because the range of successful linearisation (\( r > 0.99 \)) was often greater than with the other methods. However, method 1) was particularly useful when analysing assays specifically designed to test parallelism between unknowns and standards, since variance was smaller and more homogeneously distributed than when the logit transformation was used, resulting in a fairer, if more rigid, statistical assessment of parallelism (see later). Non-linear transformations such as the method used to analyse the radioimmunoassay (see later) yielded variable results, sometimes fitting the standard curve extremely well and other times not at all.

Validation of the Mouse Leydig Cell Assay for Use on Horse Serum

1. **Parallelism**

A fundamental requirement for a valid assay is that dilutions of unknowns must produce dose-response curves parallel to the standard curve. In these studies, parallelism was evaluated in two ways: 1) in assays
designed to test parallelism, in which 3 and 4 point dilutions of serum from horses in different physiological states were compared with the standard curve; and 2) in routine assays in which 2 point dilutions of a number of unknowns or pools of unknowns were compared with the standard curve. Statistical analysis of these two approaches differed. For approach 1), data were expressed as the square root of the amount of testosterone produced at the logarithm of the LH dose given and parallelism evaluated using standard in vivo bioassay statistics (Finney, 1961). For approach 2), routine logit-log transformation was used to gain LH potency estimates (in ng/ml serum) and parallelism evaluated within and across assays by paired t-test, for which it was assumed that if unknowns were parallel to the standard curve, the expected difference between potency estimates calculated from dilutions 1 and 2 would be zero. In routine assays, assessment of parallelism to the standard curve of individual two-point dilutions was made using the arbitrary criterion that parallelism existed if the two potency estimates differed by $\leq 20\%$. If $> 20\%$ discrepancy occurred, the estimate derived from the dilution falling in the steeper part of the standard curve was used; occasionally both estimates were discarded. (NB: This selection procedure was applied only to decide if a given value could be used in the final analysis of an experiment. All data were used to evaluate parallelism by paired t-test as described above.)

Figure 4 compares 3 and 4 point dilutions of serum with the standard curve. Analysis showed that dilutions of sera from geldings and from mares in oestrus, at various times in diestrus (days 5, 6 and 17 (mare retained corpus luteum) post-ovulation), in anoestrus, and after GnRH administration, were parallel to the standard.

Potency estimates, derived from two-point dilutions and classified on the basis of 1) physiological state of the horse from which the serum was collected and/or 2) amount of serum assayed, are tabulated in Appendix.
Figure 4: Comparison of dilutions of standard LH and sera from horses in various physiological states in the in vitro bioassay.

**Post-GnRH and Gelding Sera**

![Graph showing comparison of dilutions of standard LH and sera from horses in various physiological states in the in vitro bioassay for Post-GnRH and Gelding Sera.](image)

**Dioestrous and Anoestrous Sera**

![Graph showing comparison of dilutions of standard LH and sera from horses in various physiological states in the in vitro bioassay for Dioestrous and Anoestrous Sera.](image)
Figure 4: cont'd

![Graph showing testosterone production from Dioestrous, Oestrous, and Post-GnRH sera](image)

- **Standard**
- **Post-GnRH serum**
- **Dioestrous serum**
- **Oestrous serum**

*(LH) or µl Serum Added*
la. In each classification, paired t-test showed no reason to reject the hypothesis that dilutions were parallel to the standard curve.

2. Specificity

Van Damme et al. (1974) have reported that the bioassay is highly specific for LH (and LH-like hormones, e.g. hCG) and that human FSH, TSH, ACTH, prolactin, growth hormone, vasopressin, oxytocin, and GnRH "did not influence the bioassay method at levels likely to be found in biological samples." In the horse, only FSH, of the anterior pituitary hormones was available in sufficiently pure preparation to test cross reaction in the assay. The equine FSH used was prepared by the method of Braselton and McShan (1970) by Dr L. Nuti (University of Wisconsin, Madison, Wisconsin, U.S.A.) and contained 0.16 units NIH-LH Sl/mg by ovarian ascorbic acid depletion assay and 1.3 units/mg LH by ventral prostrate weight assay (Braselton and McShan, 1970). Cross reaction was determined by adding varying amounts of eFSH to the assay, up to 1.25 ng/tube; which at the smallest dilution of serum used in the assay (i.e., 8 μl/tube) would correspond to a serum eFSH concentration of 162.5 ng/ml, a level more than 8 times the average cycle maximum (Evans, 1977). In two separate assays, mean eFSH cross reaction was $2.2 \pm 0.05\%$ ($\bar{x} \pm SD$). Since the Braselton and McShan preparation of pure equine LH has a potency of 5.5 units NIH LH Sl/mg, the FSH preparation must contain at least $0.16/5.5 \times 100 = 2.9\%$ LH contamination. Thus, all eFSH cross reaction in the bioassay could be explained by LH contamination of the eFSH. Further confirmation of the absence of FSH cross reaction in the assay was the observation that at the time of maximal serum FSH levels in the cyclic mare (i.e., mid-dioestrus), bioassayable LH levels were minimal (see later).

Progesterone (Garfink et al., 1976; Gnodde et al., 1979) and possibly other steroids (Lichtenberg and Pahnke, 1976; Rajalakshmi et al.,
1979) have been reported to interfere in testosterone production assays, presumably by providing testosterone precursors. The effect of steroids on the present assay system was studied in two ways: 1) by removing steroids from serum by charcoal adsorption (Rajalakshmi et al., 1979), and 2) by adding progesterone (Δ⁴-pregnen-3, 20-dione, Sigma Chemical Co.) to serum in amounts up to 100 ng/ml serum and observing effects on potency estimates.

Charcoal (activated, BDH Chemicals) was washed and dried as described by Rajalakshmi (1979), a 1% (w/v) suspension in assay diluent prepared, and 0.5 ml added to 0.5 ml serum at 4°C. This mixture was vortexed and allowed to stand for 0.5 h at 4°C, then centrifuged at 1000 x g / 10 min and the supernatant removed, diluted and assayed. Table 2 compares potency estimates of various sera before and after charcoal treatment. Paired t-test showed no significant effect of charcoal treatment on the potency of sera from mares 1) in early oestrus; 2) immediately post-ovulation (day +1 or +2); 3) 10 days post-ovulation or 4) given 10 mg oestradiol benzoate and 150 mg progesterone in oil intra-muscularly daily for 6 days (t = -1.53, df = 10, n.s.).

Addition of large amounts of progesterone to serum did not alter potency estimates (one-way analysis of variance).

3. Recovery

As another test of the effect of serum components on the bioassay, known amounts of standard LH were added to 1, 2, 4, and 8 μl of anoestrous mare serum (generally samples from one of 4 mares, Coming In, Lady Sherelle, Jenny and Oriental Scott, whose mid-winter LH levels were almost immeasurably low by bioassay), assayed, and recovery determined against a "serum-less" standard curve after correction for LH content of the serum. When 1, 2 and 4 μl of serum were used, recovery of added LH was

¹At least 5 times maximum dioestrous levels (Ginther, 1979)
Table 2: Potency estimates of various sera before and after charcoal treatment.

<table>
<thead>
<tr>
<th>Serum Pool</th>
<th>Potency (ng/ml): $\bar{x}$ + S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Charcoal Treated</td>
</tr>
<tr>
<td>A = gelding</td>
<td>31.7 ± 1.9</td>
</tr>
<tr>
<td>B = gelding</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>C = early oestrus</td>
<td>50.7 ± 4.2</td>
</tr>
<tr>
<td>D = early oestrus</td>
<td>23.8 ± 0.5</td>
</tr>
<tr>
<td>E = early oestrus</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>F = post-ovulation</td>
<td>82.5 ± 5.9</td>
</tr>
<tr>
<td>G = post-ovulation</td>
<td>21.2 ± 0.5</td>
</tr>
<tr>
<td>H = P₄ + E₂ treated</td>
<td>15.7 ± 1.7</td>
</tr>
<tr>
<td>I = P₄ + E₂</td>
<td>13.1 ± 1.7</td>
</tr>
<tr>
<td>J = P₄ + E₂</td>
<td>32.7 ± 1.0</td>
</tr>
<tr>
<td>K = P₄ + E₂</td>
<td>35.3 ± 4.3</td>
</tr>
</tbody>
</table>
quantitative, being 98.5 ± 3.3% (X ± SEM) in 30 experiments (expected vs observed, least squares linear regression; slope = 1.00, intercept = 0.33, r = 0.97). However, when 8 µl of serum was used, recovery declined to 68.4 ± 3.8% (10 experiments). Figure 5 illustrates the effect of adding increasing amounts of anoestrous mare serum to the position of the LH standard curve. Analysis of variance of this experiment showed that the standard curve was affected only by the greatest amount of serum added - i.e., 8 µl (P < 0.05). However, no serum-LH interaction was observed (F_{12}^{1.335}, n.s.) indicating that the slope of the standard curve was not altered by serum. These results are somewhat contradictory to those presented earlier, which showed parallelism between the "serum-less" standard curve and serum in amounts including 8 µl. It is possible that the particular anoestrous mare serum used in the recovery experiments, which by necessity contained extremely low LH concentrations, may also have contained substances which, when in high concentration in the assay inhibited testosterone production. Because of the extreme sensitivity of the assay, recovery experiments using 8 µl of other sera, e.g. from dioestrous mares, were difficult, due to the higher LH content of these sera. Therefore, as a precautionary measure, assay of more than 4 µl of serum was avoided whenever possible. When not possible (almost always with anoestrous mares), serial 1:1 dilutions of the samples were included in the assay to check parallelism to the serum-less standard curve, and frequently a standard curve containing the same amount of serum as the unknowns was also included. It should be emphasised once again that rarely was lack of parallelism to the serum-less standard curve observed, even at the highest serum concentrations used.
Figure 5: In vitro bioassay standard curves in the presence of increasing amounts of horse serum.
4. Repeatability and Precision

These statistics were not calculated for all assays; however, in a representative sample of 44 assays (all successful mouse assays between #49 and #133, i.e. ~50% of all successful assays done) interassay coefficient of variation (SD/\bar{X} \times 100) was 15.2% as determined from repeated assay of a serum pool diluted to fall in the mid-range of the standard curve; and 18.3% as determined from repeated assay of a serum pool diluted to fall in the upper range of the standard curve. Within-assay variation over the LH range in which approximately 90% of serum samples were diluted to fall was 4.9% as determined in 14 assays from duplicate dilutions of 113 serum samples randomly placed within the assay (method of calculation from Rodbard, 1974).

The index of precision \( \lambda = \frac{SD}{\text{slope}} \) was calculated for the 25 assays in Experiments 1 and 2, mean \( \lambda \) being 0.043. This compares well with the value of 0.042 quoted by van Damme, Robertson and Diczfalusy, 1974b).

II. RADIOIMMUNOASSAY

The LH radioimmunoassay system used was first described and validated for measuring LH in horse serum by Noden et al. (1974) and later modified and validated by Evans (1977). The present system consisted of 125I-labelled ovine LH (NIH-LH-S18)*, an antibody against ovine LH (GDN 15)* and equine standards (CI 1-37). Because of recent modifications to the assay, notably use of CI 1-37 as standard and alteration in incubation schedule, a brief description of assay methodology and validation of the altered system will be given.

*Gift from National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland, U.S.A. and gift from Dr G.D. Niswender, Colorado State University, Fort Collins Co., U.S.A.
The Assay

All reagents were added to the assay in assay diluent, which consisted of 0.01 M P\textsubscript{4}O\textsubscript{4} buffer (i.e., 0.295 g/l Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 1.15 g/l Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 0.15 M NaCl, 0.1% EDTA, 0.28% egg albumen (crude powder, Grade II, Sigma Chemical Co.) and 0.08% NaN\textsubscript{3}, pH 7.35 - 7.45.

Lyophilised anti-ovine LH was reconstituted as to instructions on the bottle, and stored at -20° in 2 ml aliquots of a 1 in 40 dilution, until needed for the assay when a further dilution to 1 in 64,000 (in assay diluent) was made.

Ovine LH was iodinated to an approximate specific activity of 60 - 100 mCi/µg by a modification of the method of Landon et al. (1967) using 1 mCi Na\textsuperscript{125}I (Radiochemical Centre, Amersham, England), 20 µl 0.5 M P\textsubscript{4}O\textsubscript{4} buffer, pH 7.4, 20 µg Chloramine T in 10 µl 0.1 M P\textsubscript{4}O\textsubscript{4} buffer, pH 7.4 and 5 µg NIH LH S18 in 25 µl 0.01 M P\textsubscript{4}O\textsubscript{4}-0.14 M NaCl buffer, pH 7.0. The reaction was stopped after 35 sec by addition of 120 µg sodium metabisulfite in 50 µl 0.1 M P\textsubscript{4}O\textsubscript{4} buffer, pH 7.4 followed by 100 µl of "carrier solution", consisting of 0.05 M P\textsubscript{4}O\textsubscript{4} buffer, pH 7.4 and 0.5% bovine serum albumin. Labelled hormone was separated from unreacted iodide by gel filtration, using a 1 x 22 cm Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) column and collecting approximately 0.5 ml fractions. The column buffer consisted of 0.05 M P\textsubscript{4}O\textsubscript{4} buffer, pH 7.3 - 7.4, 0.1% bovine serum albumin, and 0.02% NaN\textsubscript{3}. A typical separation profile is shown in Figure 6. Fractions in the peak of protein-bound radioactivity were pooled and stored in small aliquots at -20°. The labelled hormone was generally repurified before use in the assay by gel filtration as described above, except that the column buffer consisted of 0.01 M P\textsubscript{4}O\textsubscript{4} buffer, pH 6.8 - 7.0, 0.15 M NaCl, 0.02% NaN\textsubscript{3} and 1.5% bovine serum albumin. Hormone was iodinated at 2 - 3 week intervals and labelled hormone older than 3 weeks was not used.
Figure 6: Typical elution pattern of freshly iodinated ovine LH from a 1 x 20 cm Sephadex G-100 Column.

* Fractions kept for use in assay.
Assay diluent, serum, anti-ovine LH and $^{125}$I-labelled LH were added to assay tubes by a highly precise and repeatable automatic diluting pipette (Micromedic Systems, Inc., Model 2500, Philadelphia, Penn., U.S.A.).

Incubation schedule was modified to that recommended by Hunter and Bennie (1979) as giving greatest specificity to human gonadotrophin radio-immunoassays and was as follows:

1) Day 1: Unknowns/standards and anti-oLH serum combined and incubated 16 - 20 h at room temperature.

Standards were 0, 0.156, 0.312, 0.625, 1.25, 2.5, and 5.0 ng CI $^{1-37}$/assay tube and were added to the assay in 200 μl of assay diluent. Generally, 20 - 50 μl of serum was needed for assay, and was added to 200 μl of assay diluent. An equivalent amount of low LH mare serum (generally, a pool of anoestrous mare and late pregnant (> 300 d) mare sera) was added to all standard tubes. Finally, 200 μl of anti-oLH serum (1 in 64,000 dilution) was added to all tubes, except "blank" tubes (to which 200 μl assay diluent was added to 200 μl diluent plus the appropriate amount of serum; these tubes were used to calculate non-antibody-bound counts in the final precipitate), and "total counts" (empty at this point). The assay was incubated in the dark, in a small windowless room in which temperature was reasonably constant throughout the year.

2) Day 2: Assay brought to $4^\circ$ and 18,000 - 25,000 cpm $^{125}$I-oLH added to all tubes in 200 μl assay diluent. Assay incubated at $4^\circ$ for 24 - 48 h.

Added with the $^{125}$I-oLH was 0.7 μl/tube of non-immune rabbit serum.

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1 The machine was loaned to the Veterinary Department by the Endocrinology Unit, the Princess Margaret Hospital, Christchurch. Use of the machine was deeply appreciated by the author.
3) Day 3 or 4: 0.41 units goat anti-rabbit gamma globulin (Calbiochem, San Diego, Calif., U.S.A.) added to all tubes except total counts and assay incubated 16 - 24 h at 4°.

4) Day 4 or 5: Antibody-bound hormone separated from free hormone. This was achieved by centrifuging assay tubes at 1500 x g for 20 min at 4°, after which the supernatant was removed from the pellet by decanting. Before centrifuging, 2 ml of "spinning down buffer" consisting of 0.01 M PO₄, 0.1% EDTA, 0.28% egg albumen, and 0.08% NaN₃, pH 7.4 (Evans, 1977) was added to all tubes (except total counts) to dilute the amount of radioactivity in any supernatant remaining with the antibody pellet. The pellets were then counted in a standard gamma counter.

Assay Design

The most common design used was one-point assays of unknowns in duplicate. Occasionally, unknowns were also assayed at a 1:1 dilution when the possibility of non-parallelism between unknown and standard curve existed (e.g., if the sample had been collected immediately after GnRH administration). Standards were assayed in quadruplicate. Immediately after addition of serum to the assay, the tubes were completed randomised according to a table of random numbers. This step was precautionary to prevent bias in results due to position of samples in the assay. As an additional quality control measure, duplicate "zero" tubes (diluent, anti-oLH, 125I-oLH and anti-rabbit gamma globulin) were placed at 50-tube intervals to check "drift" in antibody binding throughout the randomised assay. Tubes were restored to numerical order just before counting.
Assay Analysis

Results were calculated by a computer program (Burger et al., 1972) for a least squares fit of the standard curve to the equation

\[ Y = \frac{A}{(C + X^E)} \]

where \( Y \) = counts antibody bound, \( X \) = LH concentration, and \( A, C \) and \( E \) = constants determined by the computer program. Burger's program was modified to correct for the presence of non-antibody-bound counts in the pellet in a way similar to that described for the comparable calculation in the testosterone assay. Briefly, it was assumed that the ratio non-antibody-bound counts:free counts was a constant that could be determined from counts present in "blank" tubes (i.e., tubes containing all reagents BUT antibody).

The computer program also calculated the precision of measurement along the standard curve and the standard error of the sample potency estimate.

Validation of Modified Assay Procedure

Quantitative recovery of LH added to serum and specificity have already been demonstrated in this assay system by Evans (1977), as well as parallelism between dilutions of serum and equine pituitary LH. However, since a different preparation of equine pituitary LH was used in these experiments, it was necessary to show parallelism between serum dilutions and the new standard. This problem was approached, as with the bioassay, in two ways: 1) in assays specifically designed to test parallelism in which 3-point dilutions of serum from horses in various physiological states were compared with the standard curve, and 2) in routine assays in which 2-point dilutions of unknowns were compared with the standard curve. Statistical analysis of the 2 approaches differed.
Approach 1): Because the computer program used to analyse the immunoassay did not linearise the dose-response curve, traditional methods for evaluating parallelism could not be used. Table 3 shows potency estimates \( + \) standard error of 3 serial dilutions of various sera. LH concentration in ng/ml was calculated from each dilution for each sample and these data submitted to two way analysis of variance in which treatment was dilution and serum samples were replications. Analysis showed that dilution did not affect potency estimates \( F_{16}^2 = 0.32, \text{n.s.} \), and therefore parallelism between serum samples and standards was assumed.

Approach 2): As with the bioassay, parallelism between 2-point dilutions of serum and standards was evaluated by paired t-test. Potency estimates derived from 2-point serum dilutions and classified on the basis of the physiological state of the horse from which the serum was collected are shown in Appendix 1b. In each classification, paired t-test showed no reason to reject the hypothesis that dilutions were parallel to the standard curve.

Repeatability was not calculated for all assays; however, in a representative sample of 50 assays (all successful assays between \#14 and \#73 containing replicate standards, i.e. ~ 60\% of all successful assays done), interassay coefficient of variation \( \frac{\text{SD}}{\bar{x}} \times 100 \) was 12.1\%, as determined from repeated assay of a low LH serum pool - and 12.4\%, as determined from repeated assay of a high LH serum pool. Within-assay variation over the LH range in which approximately 85\% of serum samples fell was 3.4\% as determined in 7 assays from duplicate measurements of 68 serum samples randomised through the assay (method of calculation from Rodbard, 1974).
Table 3: Immunoactive LH concentration (+ standard error) in 3 serial 1:1 dilutions of various sera.

<table>
<thead>
<tr>
<th>State of Horse</th>
<th>Serum Added (µl)</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>dioestrus</td>
<td>2.1 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>11.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>dioestrus</td>
<td>2.9 ± 0.1</td>
<td>5.8 ± 0.2</td>
<td>12.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>dioestrus</td>
<td>6.8 ± 0.4</td>
<td>16.6 ± 0.7</td>
<td>30.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>post GnRH</td>
<td>1.1 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>post GnRH</td>
<td>14.1 ± 0.6</td>
<td>29.3 ± 1.3</td>
<td>56.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>oestrus</td>
<td>17.8 ± 0.7</td>
<td>35.1 ± 1.6</td>
<td>67.0 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>gelding</td>
<td>6.7 ± 0.2</td>
<td>12.1 ± 0.5</td>
<td>25.4 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>gelding</td>
<td>3.8 ± 0.1</td>
<td>6.7 ± 0.2</td>
<td>14.3 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>
III. EQUINE LEYDIG CELL ASSAY

LH receptors are probably not identical on horse and mouse gonadal cells. Thus, the mouse Leydig cells used in the in vitro bioassay might not bind or respond to various forms of LH in the same way horse cells would, thereby providing inaccurate estimates of samples' biological activity in the horse. For this reason, attempts were made to develop an in vitro bioassay for LH using equine Leydig cells. These attempts were largely unsuccessful, with only 5 assays (all performed between October and December, 1979) offering encouraging results. Protocol for and results from these "successful" assays will be presented below, as well as results of investigations into reasons for failure of subsequent assays.

MATERIALS AND METHODS

Testes were from horses ≤ 2 years of age of various breeds (pony, thoroughbred, standardbred) and were collected by local veterinarians. "Successful" assays used testes from yearlings or younger; small (approximately 2 x 1 cm) immature testes yielding best results. Immediately on removal, testes were placed in ice cold isotonic saline, sliced in several places to allow quick cooling and rushed on ice to the laboratory. Here, interstitial cells were dispersed and the testosterone production assay performed using the method described by Davies et al. (1979) for human testis. Briefly, testes were decapsulated, cut into approximately 1 g chunks, and incubated in tissue culture medium 199, pH 7.3 - 7.35, containing 1 mg/ml BSA and 1 mg/ml collagenase (Worthington Type 1, Sigma Chemical Co.) for 10 min in a shaking water bath at 35°C (ratio M199:testis = 1 ml:1g). The M199 was bicarbonate buffered and this incubation was performed under 5% CO₂ in O₂. After incubation, the testis chunks were diluted with 5 volumes isotonic saline, shaken several
times and freed cells separated from chunks by filtration through several layers of gauze bandage. Only partial dissociation of chunks into seminiferous tubules and interstitial cells was achieved by this procedure; mean yield of interstitial cells in successful assays being $1.9 \times 10^6$ cells/g testis (range = $1.2 - 3.4 \times 10^6/g$). As with the mouse in vitro bioassay, vigorous attempts to identify cell types were not made; however, histologically most cells appeared to be Leydig cells (Dellman and Brown, 1976); many, especially in younger testes, having yellow pigmented foamy cytoplasm. When tested by trypan blue exclusion, 40 - 50% of these cells appeared to be viable. Red blood cells and spermatozoa were always present in cell preparations; spermatozoal contamination increasing with testis size (and presumably maturity). After separation from chunks, dispersed cells were sedimented by centrifuging at 1000 x g for 10 min at 4°, resuspended in M199 - 0.1% BSA (1 ml/g testis collagenase digested) and incubated under 5% CO$_2$ in O$_2$ for 1 h at 17° in a shaking water bath. Cells were then sedimented as described above and placed in M199 - 0.1% BSA, containing 100 U/ml heparin (Grade I, Sigma Chemical Co.) and 0.125 mM isobutyl-methyl xanthine (MIX, Sigma Chemical Co.). The ratio M199:weight testis collagenase digested was 3 ml:lg; giving a mean cell concentration of 630,000/ml (range = 400,000 - 1,100,000 cells/ml). One ml of this cell preparation was added to 10 ml glass vials containing 1.1 ml standard LH or unknown in M199 - 0.1% BSA. The amount of LH per vial ranged from 0 to 5.6 ng CI 1-37. The selection of 5.6 ng as top standard was based on results of the first "successful" equine assay in which 1.68, 16.8 or 168.0 ng CI 1-37 or 0.15, 1.5 or 15 IU human chorionic gonadotrophin (hCG, "Chorulon", Intervet, Boxmeer, Holland) had been added to cells. Maximal stimulated secretion occurred at 1.68 ng CI 1-37 or 0.15 IU hCG. The serum samples in the "successful" assays were 4 pools, collected from mares in early or late oestrus, day 10 of dioestrus, or after GnRH administration. These were times at which the ratio of mouse biological:
immunological LH activity in serum differed markedly (see later). The amount of serum assayed ranged from 20 - 100 µl/vial. The assay was incubated under 5% CO₂ in O₂ for 3 h at 37°C in a shaking water bath. After incubation, vials were placed in an ice bath, the contents transferred to Wasserman tubes and cells pelleted by centrifuging at 1000 x g for 10 min at 4°C. The supernatant was removed, heated to 80°C/1 h and a portion assayed for testosterone, as described for the mouse bioassay. Occasionally, the supernatant was also assayed for androstene-dione and/or total oestrogens. The procedure for the androstene-dione assay was patterned after that for testosterone; the antibody¹ used cross-reacting 5% with dehydro-isandrosterone-dione, 3.3% with DHAS, 2.4% with testosterone and < 1% with oestrone.

The total oestrogen assay used a modification of the method of Palmer and Terqui (1977) in which oestrogen sulfates and glucuronides were hydrolysed by incubation with a crude preparation of limpet (Patella vulgata) digestive enzymes (prepared in the laboratory by the method of Levvy, Hay and Marsh, 1957). Free (i.e., unconjugated) oestrogens were then extracted with ethyl acetate and radioimmuno-assayed, using 3H-oestrone tracer (2,4,6,7 ³H-oestrone, Amersham, Batch 12), oestrone standards and an antiserum² made in guinea pigs against a mixture of oestrone, oestradiol and oestriol. The antiserum was not completely characterised; however, under assay conditions at a final dilution of 1 in 6000 it specifically bound 4.9 ± 0.7 pg 3H-oestrone out of 29.3 ± 1.9 pg added (X ± SD, n = 4 assays) and 8.9 ± 2.0 pg 3H-oestradiol out of 33.0 ± 8.5 pg added (n = 3 assays). Surprisingly, cross reaction with androstene-dione was 100%. The assay was successfully applied in the laboratory for mid-late pregnancy diagnosis in mares.

¹Gift from Ruakura Agricultural Experimentation Station.
²Gift from Dr J. Evans, Christchurch Women's Hospital.
Results

Figure 7 shows the standard curve from the best equine Leydig cell assay. In this assay, the top LH standard, 5.6 ng/vial CI 1-37, stimulated secretion of 58.0 ng testosterone compared with basal secretion (no LH added to vial) of 10.1 ng testosterone. In the other 4 successful assays, secretion stimulated by 5.6 ng LH ranged from 1.3 - 4.5 times basal rate. By comparison, maximal stimulated testosterone secretion in mouse Leydig cell assays was at least 10 times and commonly 30 - 40 times basal secretion. Basal and stimulated androstene-dione and/or oestrogen secretion were measured in 3 "successful" equine assays. Results are shown in Table 4.

Table 4: Steroid secretion by equine Leydig cells.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Testosterone</th>
<th>Androstene-dione</th>
<th>Oestrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>basal +5.6 ng LH</td>
<td>basal +5.6 ng LH</td>
<td>basal +5.6 ng LH</td>
</tr>
<tr>
<td>1</td>
<td>10.1</td>
<td>58.0</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>4.7</td>
<td>21.0*</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>4.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Secretion stimulated by 15 IU hCG

In assay 1, the major steroid product of LH stimulation was testosterone, whereas in assays 2 and 3 the major product was probably an oestrogen (note the low level of androstene-dione secretion in assay 3. The testes used in the 3 assays were from yearlings; however, colt 1 was a pony and colts 2 and 3 horses. Thus, the differences in steroids secreted in response to LH may reflect breed differences.
Figure 7: Standard curve of the best equine in vitro bioassay.
Table 5 compares mean potency estimates for 4 horse serum pools in equine and mouse *in vitro* bioassays. The 2 methods of assay ranked the potency of the pools identically and the ratio between activity in horse and mouse assays was very similar in all but the dioestrous pool, the LH levels in which were at the limit of detection of the horse assay.

**Further Experiments**

With these encouraging results, the equine Leydig cell assay was not attempted again until May, 1980. In this and subsequent assays, added LH increased cell steroid secretion very little or not at all from basal levels. Sometimes, basal testosterone secretion was moderate to high (10 - 20 ng) and at other times immeasurably low; cells always appearing equally viable. Searching for reasons for assay failure, it was observed that in May 1980, bicarbonate buffered M199 had been replaced by HEPES-buffered Eagle's minimum essential medium, the 5% CO₂ in O₂ cylinder having been returned to the factory, and that a new type of collagenase was being used (Type II, Sigma Chemical Co.). Furthermore, winter was non-breeding season for horses, and it was possible that Leydig cell responsiveness to LH stimulation was reduced compared to summer levels. (Harris, 1980). When assay failure extended into the breeding season, this latter explanation was rejected. The assay was not improved by changing the collagenase type used (to Type III, Worthington Biochemical Corp., Freehold, N.J., U.S.A.), supplementing collagenase with trypsin (62.5 mg/10 g testis, GIBCO) or hyaluronidase (1500 U/10 g testis, Hyalase® Fisons Ltd, Loughborough, Leics, England) or omitting collagenase altogether (cells washed from finely shaved testis slices were almost always dead). Increasing cell numbers (up to $6 \times 10^6$/vial) increased basal steroid production but did not result in a response to added LH. Increasing the maximum amount of LH added (to 168 ng) or substituting 15 IU hCG for CI 1-37 did not stimulate cell steroid secretion. Returning to tissue
Table 5: Potency estimates (ng/ml, $\bar{X} \pm$ S.E.M.) for 4 horse serum pools in horse and mouse in vitro bioassays.

<table>
<thead>
<tr>
<th></th>
<th>Oestrus</th>
<th></th>
<th>Dioestrus</th>
<th></th>
<th>Post-GnRH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Late</td>
<td>Day 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse assay</td>
<td>17.7 ± 5.1(3)</td>
<td>31.9 ± 0.6(3)</td>
<td>9.9(1)</td>
<td></td>
<td>30.8(1)</td>
</tr>
<tr>
<td>Mouse assay</td>
<td>23.6 ± 0.9(2)</td>
<td>48.8 ± 1.8(2)</td>
<td>8.0 ± 0.3(3)</td>
<td></td>
<td>44.1 ± 0.9(2)</td>
</tr>
<tr>
<td>Horse assay</td>
<td>0.75</td>
<td>0.65</td>
<td>1.24</td>
<td></td>
<td>0.70</td>
</tr>
<tr>
<td>Mouse assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
culture medium 199 (HEPES buffered) or changing the buffer back to sodium bicarbonate and incubating the assay under 5% CO₂ in air did not alter results, nor did bubbling O₂ through bicarbonate or HEPES buffered cells. Reasoning that assay failure could be explained by interference with LH binding to receptors, a radioreceptor assay using homogenates of horse testis was developed to test this possibility and to look for ways to improve receptor binding that could be applied to the in vitro bioassay. The method followed was adapted from that of Dr D.N. Ward (M.D. Anderson Tumor Institute, University of Texas, pers. comm.). Briefly, decapsulated horse testes were homogenised in 0.025 M TRIS (2-amino-2(hydroxymethyl)propane-1:3-diol)-HCl buffer, pH 7.2, containing 6 mM MgCl₂·6 H₂O (buffer described by Cheng, 1975), using a Sorvall Omni-mixer at top setting for 3 x 10 sec bursts. The ratio buffer:testis was 10 ml:1 g. Homogenised testis was filtered through 4 layers of gauze bandage and centrifuged at 1500 x g for 15 min at 4°C. This pellet was discarded and the supernatant poured into weighed centrifuge tubes and spun at 15,000 x g for 15 min. The pellet was retained and resuspended in TRIS buffer to a concentration of 25 mg pellet/200 μl buffer. 200 μl of this preparation was incubated in Wasserman tubes with various amounts of equine LH (4.2 - 16.8 ng/tube CI 1-37) or excess hCG to determine non-specific binding (10 - 20 IU Chorulon) in 300 μl TRIS buffer for 1.75 h at 37°C in a shaking water bath. Labelled hCG¹ (¹²⁵I = label) was added to all tubes for the last 20 min of incubation (50,000 cpm in 50 μl TRIS buffer was added). To precipitate the testis homogenate after incubation, 1 ml of 15% polyethylene glycol 6000 (BDH Chemicals Ltd) in TRIS buffer was added and tubes

¹This highly purified hCG was a gift from Dr R.E. Canfield (Columbia University) to Mr C.M. Lengoc. The hormone used in these studies was iodinated to a specific activity of approximately 24 μCi/μg by Mr Lengoc, using 0.25 mCi Na¹²⁵I (see Lengoc and Irvine, 1980, for method).
centrifuged at 1500 x g for 20 min at 4°C. The supernatant was removed by aspiration, the pellet washed once with 1 ml ice cold TRIS buffer, and then counted in a standard gamma counter.

In 5 assays, the per cent $^{125}$I-hCG specifically bound to the pellet in the absence of added hormone

\[
\frac{\text{total counts pellet bound} - \text{counts bound in presence of excess hCG}}{\text{total counts added} \times 100}
\]

ranged from 8.7 - 29.4%; the ratio of counts nonspecifically:specifically bound ranging from 0.25 to 1.4. No relationship between testis age or size and quality of assay results could be detected (see Table 6).

Figure 8 compares the displacement of $^{125}$I-hCG by equine LH from homogenates of 3 different testes. Again, results were similar and independent of testis age or size. When the radioreceptor assay was run in tissue culture medium (HEPES buffered M199) specific binding was reduced to approximately half that in TRIS (14.3% vs 8.6%, TRIS vs TCM, mean of 2 assays), without affecting the ratio counts nonspecifically bound

\[
\frac{\text{counts nonspecifically bound}}{\text{counts specifically bound}}
\]

(0.9 vs 1.0, TRIS vs TCM). These results suggested that an alternative to TCM in the equine bioassay should be found. Since the TRIS buffer used in the receptor assay was not isotonic to Leydig cells, a TRIS buffered balanced salt solution was selected (TBSS, 90 mM NaCl, 3.5 mM KCl, 1mM CaCl$_2$, 10 mM MgSO$_4$·7H$_2$O, 0.23 mM Na$_2$HPO$_4$·2H$_2$O, 0.29 mM KH$_2$PO$_4$, 16 mM TRIS, 5mM citric acid·H$_2$O, 10 mM glucose, pH 7.3; Paul (1970)). TBSS could be substituted for tissue culture medium in the mouse Leydig cell assay with little effect on basal or maximum stimulated testosterone secretion (TCM, no LH = 102 ± 78 pg testosterone, 100 pg LH = 1008 ± 342 pg; TRIS, no LH = 83 ± 11 pg, 100 pg LH = 924 ± 355 pg, X ± SD, mean of 2 assays). However, when tested first in the horse radioreceptor assay, TBSS, like TCM, reduced specific binding to receptors (assay 1: 11.9% vs 6.3%, TRIS vs TBSS; assay 2: 29.4% vs 24.1%). In assay 2, an equine LH standard curve was included and its slope was markedly flattened by TBSS (logit-log dose slope: + TBSS = -0.4, - TBSS = -2.6). Reflecting these results, little improvement in the
Table 6: Per cent $^{125}$I-hCG specifically bound (SB) and ratio of counts nonspecifically:specifically bound (NSB:SB) in radioreceptor assays using homogenates of 5 different horse testes.

<table>
<thead>
<tr>
<th>Testis Code</th>
<th>Description of Donor</th>
<th>% SB</th>
<th>NSB:SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>B*</td>
<td>12 month old pony testes 4 x 7 cm, weight = 100 g/testis, homogenate stored frozen and thawed for assay.</td>
<td>8.7</td>
<td>0.25</td>
</tr>
<tr>
<td>D*</td>
<td>Yearling horse, testes 1.8 x 2.4 cm, weight = 30 g/testis; homogenate used fresh in assay.</td>
<td>11.8</td>
<td>1.4</td>
</tr>
<tr>
<td>E*</td>
<td>2 year old horse, testis weight = 163.7 g; homogenate used fresh in assay.</td>
<td>16.8</td>
<td>0.33</td>
</tr>
<tr>
<td>F</td>
<td>11 month old horse, testes 1.8 x 2.4 cm, weight = 30 g/testis, homogenate used fresh in assay.</td>
<td>29.4</td>
<td>0.31</td>
</tr>
<tr>
<td>G</td>
<td>Pool of 5 pairs of horse yearling testes, weight range = 30 - 60 g/testis, homogenate used fresh in assay.</td>
<td>11.9</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*Assays run together using same reagents.
Figure 8: Displacement of $^{125}\text{I-hCG}$ by equine LH in homogenates from 3 different horse testes.
equine in vitro bioassay was observed when TBSS was substituted for TCM.

As another approach to the problem, radioreceptor assays using equine cells dispersed by gentle agitation of small chunks of testis were attempted in TRIS, TBSS and TCM with or without HEPES buffer. Sucrose (0.3 M) was added to the TRIS buffer in an attempt to make the buffer isotonic \(^1\) for Leydig cells. In the 5 experiments done, the number of cells added ranged from 3.2 to 13.8 \(\times 10^6\) /tube. Very little specific binding of \(^{125}\)I-hCG by cells was observed in any experiment; however, slightly more binding/\(10^6\) cells occurred in TRIS-sucrose than other buffers: \[
\frac{\text{counts specifically bound}}{10^6 \text{ cells}} = \text{TRIS-sucrose, 0.61%}; \quad \text{TCM + HEPES, experiment 1, 0% (6.8 - 13.6 \(\times 10^6\) cells added), experiment 2, 0.5%}; \quad \text{TBSS, 0% (6.5 \(\times 10^6\) cells added)}.\]

Although 6 \(\times 10^6\) Leydig cells represent 1.5% of total Leydig cells in a 30 g colt testis (calculations based on values given by Johnson and Neaves, 1981), it is possible that the number of LH receptors present in the assay was considerably less than when 25 mg of a homogenate consisting largely of cell membranes was added.

Having concluded that TBSS and TCM reduced but did not prevent LH receptor binding in horse testis homogenates and possibly in dispersed Leydig cells, it was decided to change the line of attack to one suggested by Labruzzo et al. (1980) who reported that testosterone secretion by small chunks of horse testis (~ 600 mg) could be stimulated by incubation with 100 IU hCG. Following Labruzzo's method, but using only 160 mg of tissue in TBSS and 10 U hCG or 16.8 ng eLH, it was observed that chunk testosterone secretion was stimulated 2.0-fold over basal secretion by hCG and 2.6-fold by eLH. However, replication of results was very poor, even though chunks were weighed to allow correction for differences in size and the assay was not sensitive enough to measure serum LH levels. Nevertheless, results suggested that cell dispersal was also a factor in assay failure. Partially homogenising testes into a slurry of pipettable cell

\(^1\)Actually calculations went slightly astray and this TRIS-sucrose buffer was hypertonic (approximately 400 mOsm cf 280 for "physiological saline").
cell clumps (50 - 60% alive by trypan blue exclusion) did not improve the assay, nor did the addition of prostaglandin synthetase inhibitor (2.5 mg/50 ml TBSS, flunixin meglumine, FINADYNE®, Vetco Products Ltd, Auckland) to collagenase dispersed cells. Fuchs and Chantharaksri (1981) showed that LH stimulated but not basal testosterone production by rat testis *in vitro* was inhibited by prostaglandin F₂α and enhanced by addition of a prostaglandin synthetase inhibitor (diclofenac sodium). It was possible then that disruption of horse testis caused prostaglandin synthesis and/or release, which contributed to assay failure. However, if this were the case, flunixin at the concentration used did not remedy the problem.

In March 1982, attempts to develop an equine Leydig cell *in vitro* bioassay were reluctantly halted. After 3 years of experiments, reasons for the assay's initial success seemed less understandable than those for subsequent failures. Results suggested cell dispersal to be a delicate step in the procedure, and it is possible that type and/or batch of collagenase used were critical for assay success. Reports of the effectiveness of collagenase in successfully dispersing various tissues have been contradictory (Paul, 1970), probably because of difficulty in producing a standard collagenase preparation and controlling the activity levels of contaminating enzymes (Sigma Chemical Co. Catalogue, 1982). Alternatively, it is possible that a potentially successful remedy was applied to testes that would have been unresponsive under ideal conditions.
CHAPTER II.

BIOACTIVE AND IMMUNOACTIVE LH LEVELS IN SERUM OF CYCLIC AND SEASONALLY ACYCLIC MARES
The purpose of these experiments was to measure serum LH levels through the mare's oestrous cycle by in vitro bioassay and then to compare biological LH activity with immunological.

A. EXPERIMENTAL ANIMALS

The 14 mares used in these Experiments 1 and 2 were 400 - 600 kg Standardbreds, 3 - 14 years of age (see Appendix 2 for individual description of these mares). The mares were kept in groups in paddocks at one of three locations within a 20 km radius of Lincoln College: 1) Lincoln College Research Farm, 2) a commercial Standardbred breeding farm (Nevele R Stud, Prebbleton), or 3) the author's 15 acre property (see Appendix 2). During the experiments, all mares were maintained solely on mixed sward pastures. All except two mares were non-lactating. Mares were "teased" to determine sexual receptivity (oestrus) daily at locations 1 and 3, and every other day at location 2 until oestrus was detected, after which mares were teased daily for the rest of the experiment. Teasing techniques were similar at the 3 locations. The stallion approached the mares across a gate (locations 1 and 3) or teasing crush (location 2) and was allowed to smell, nip, and nuzzle the mares, but not to mount. Mares were considered to be in oestrus if they displayed several of the behavioural responses to the stallion described by Ginther (1979), e.g. clitoral winking, urinating, posturing, absence of hostility (see Gallagher, 1975, for further details). When the response of a mare to teasing was ambiguous, her ovaries were palpated per rectum and/or serum progesterone levels assayed.
B. EXPERIMENTAL PROTOCOL

Experiment 1

Seven mares were bled once daily through an oestrous cycle (mean cycle length $\pm$ SD = 23 $\pm$ 2 days). Ninety per cent of the daily blood samples were collected between 0800 and 1200 h. The oestrous cycles studied occurred between December and June. At approximately this latitude, the peak of the breeding season as indicated by greatest frequency of ovulation is in January - February. All of the 4 cycles occurring during April or later began and ended with spontaneous periods of behavioural oestrus and 2 of these were followed by at least 2 more apparently normal cycles (Mindanao and Orient Star). Of the 2 mares remaining (Descent and True Return), Descent was not teased regularly after the studied cycle and True Return began another experiment during which a moderate dose of GnRH was given daily for 30 days. One mare, Mindanao, was studied both in January and June to examine the effect of season on individual LH patterns. Thus, experiment 1 consisted of 8 cycles from 7 mares. Serum LH levels were measured by radioimmunoassay and in vitro bioassay.

Experiment 2

Eight mares were bled 3 times daily at approximately 6 h intervals from the first day of oestrus through the first day of dioestrus (mean length of oestrus $\pm$ SD = 4 $\pm$ 1 day). The cycles studied occurred between December and February. Serum LH was measured by radioimmunoassay and in vitro bioassay.

---

1 Unpublished observations from studs in the area, and consistent with the report of Osborne (1966).
Experiment 3: Aim: to Compare Bio- and Immuno-active LH Levels in Seasonally Acyclic Mares

Bio- and immuno-active LH levels were measured in 18 serum samples collected from 8 seasonally acyclic horse mares (see Appendix 2) in the course of several experiments. Mares were deemed to be seasonally acyclic on the basis of criteria similar to those described by Evans and Irvine (1977), i.e. the presence of small, hard, inactive ovaries as determined by rectal palpation, serum progesterone levels < 1 ng/ml and, in some instances, induction of oestrous behaviour approximately 16 h after intramuscular injection of 1.5 mg oestradiol benzoate. Samples were selected for inclusion in this survey because LH assays had been done in the same batch as those on experiment 1 samples and thus potency estimates should be directly comparable.

In these and all subsequent experiments (except where noted), blood was collected by jugular venepuncture and kept at 4°C overnight, after which serum was recovered by centrifugation. Each sample was stored at -20°C in two separate aliquots so that both bio- and immuno-assays could be done on once-thawed serum.

STATISTICAL ANALYSIS

Cycles were normalised to the first day of dioestrus (= Day 1). It was felt that termination of sexual receptivity would provide as good if not better indication of physiologically significant changes in steroid environment than time of ovulation. In experiment 1, daily mean bio- and immuno-active LH levels were compared by paired t-test (using statistical programmes, HP 41CV handheld calculator). For both experiments, the ratio of biological:immunological (B:I) activity in each serum sample was calculated. Changes in the ratio during the studied period were analysed, after logarithmic transformation (a log-normal distribution of the ratio...
was assumed (Gaddum, 1945; see also Romani et al., 1977) by two-way analysis of variance with day of cycle as treatment and individual mares as replications. For the purpose of analysis of experiment 2, the 3-daily samples were considered to give 3 estimates of a single daily mean B:I ratio. In both experiments, daily means were compared with Duncan's new multiple range test (Steel and Torrie, 1980) after analysis of variance indicated a significant F for treatment. To study the effect of season on cycle B:I ratio, experiment 1 cycles were arbitrarily classified as "mid-season" (completed earlier than 31/3, n = 4) or "late-season" (completed after 31/3, n = 4). Analysis of variance was performed on the logarithms of the B:I ratios. Treatments were considered to be season (mid or late) and day of cycle; the interaction between season and day of cycle was also analysed.

For acyclic mares sampled more than once, mean bio- and immuno-active LH levels were calculated. Mean LH levels were also calculated for each experiment 1 mare for the period between days 7 and 14 of dioestrus. This period was selected because, by both methods of assay, LH levels were stable and low (see Figure 9). Acyclic and dioestrous LH levels were compared by Student's t-test (41CV hand-held calculator). Bio- and immuno-assay data was analysed separately.

For acyclic mares sampled more than once, geometric mean B:I ratio was calculated. Geometric mean B:I ratio was also calculated for each experiment 1 mare for the dioestrous period defined above. Dioestrous and acyclic B:I ratios were compared, after logarithmic transformation, by Student's t-test.

The analyses of variance were performed by the Lincoln College computer, using the general statistical program (GENSTAT V) developed at the Rothamsted Experimental Station.
RESULTS

Levels of bio- and immuno-active LH during each of the 8 cycles in experiment 1 and the 8 oestruses in experiment 2 are tabulated in Appendix 3. Mean bio- and immuno-active LH levels (+ S.E.M.) during the 8 experiment 1 cycles are shown in Figure 9. The patterns of LH measured by the two methods were similar throughout the cycle, except that bioactive LH rose earlier in the periovulatory surge and was significantly higher on Days -6, -4, -2 and -1 than immuno-active LH (P < 0.05). The geometric mean B:I ratio during the grouped cycles was 1.15 (0.98:1.32, 99% confidence limits). However, this ratio varied significantly with day of cycle (P < 0.001) being high as LH levels increased during oestrus, but dropping sharply on the last day of oestrus to remain stable and low during dioestrus. A similar and significant (P < 0.001) change in B:I ratio was seen in experiment 2 (see Figure 10). Furthermore, 6-hourly blood samples showed that the late oestrus-early dioestrus decline in B:I ratio occurred smoothly in individual mares (see Figure 11 and Appendix 3). Analysis of variance indicated that there was significant (P < 0.001) variation between mares in B:I ratio in both experiments; cycle means in experiment 1 ranged from 0.63 to 1.98. Mares also appeared to differ in the extent and duration of change in B:I ratio during the cycle. Part of this variability could be attributed to the significant effect of season on cycle B:I ratio (P < 0.005). Mean B:I ratio during mid-season cycles was 1.01 while during late season cycles it was 1.32 (least significant difference = 0.073). No interaction between season and day of cycle could be demonstrated ($F_{22}^{125} = 0.42$, n.s.), implying that the pattern of change in ratio was similar in mid- and late season. However, comparison of graphs of daily mean B:I ratio during the grouped cycles from each of the two periods suggested that the extent of the late oestrous/early dioestrous decline in ratio was greater in mid-
Figure 9: Mean LH levels measured by radioimmunoassay and in vitro bioassay throughout the oestrous cycle in 8 mares. Day 1 = first day of dioestrous. Bars represent the standard error of the mean. Starred bioassay values are significantly different to corresponding immunoassay values at the 0.05 level. Also shown is the daily geometric mean ratio of biological: immunological (B:I) LH activity in serum of the same 8 mares throughout the oestrous cycle. Values without a common subscript are significantly different at the 0.01 level.
Figure 10. Daily geometric mean ratio of biological : immunological LH activity in serum of 7 mares bled 3 times daily from the first day of oestrus through the first day of dioestrus (Experiment 2). Values without a common subscript are significantly different at the 0.01 level.
Figure 11. Ratio of biological : immunological LH activity in serum of 3 mares bled 3 times daily from the first day of oestrus through the first day of dioestrus (Experiment 2).
than late season (see Figure 12). This observation is supported by comparison of the pattern of B:I ratio during January and June cycles of the same mare, Mindanao (see Figure 12). One experiment 2 mare, Tact Del, who had 13-day cycles, failed to show any change in B:I ratio. Progesterone assay showed a transient increase in serum levels during the first few days of dioestrus with a sudden fall on the tenth day of dioestrus as the mare returned to oestrus. Despite repeated matings, this mare failed to conceive. Because of Tact Del's abnormality, her data were omitted from analysis. Mean LH levels in acyclic mares were significantly lower than dioestrous levels, regardless of method of assay (X ± SEM Bioassay: Acyclic = 3.6 ± 0.5 ng/ml, dioestrous = 9.8 ± 2.8 ng/ml, t = 2.15, P < 0.05; RIA: Acyclic = 5.1 ± 0.7 ng/ml, dioestrous = 8.4 ± 1.2 ng/ml, t = 2.35, P < 0.05). On the other hand, geometric mean B:I ratio in acyclic mare serum was slightly but not significantly lower than mean ratio in dioestrous serum (acyclic = 0.62, dioestrous = 0.98, t = 1.22, n.s.).
CHAPTER III.

INVESTIGATION OF FACTORS AFFECTING THE RATIO OF BIOLOGICAL TO IMMUNOLOGICAL LH ACTIVITY IN MARE SERUM
Figure 12: Daily geometric mean B:I ratio in mid-season and late-season cycles.

Mindanao - Daily B:I ratio in January and June cycles.

Day of Cycle
(Day 1 = first day of dioestrus)
The purpose of these experiments was to study the effect of GnRH and ovarian steroids on biological and immunological LH activity in serum; the possibility of interaction between releasing hormone and ovarian steroids in regulating both nature and levels of serum LH was also investigated. This section deals primarily with effect of treatment on the nature of serum LH. Regulation of LH levels is considered further in Sections 2 and 3.

MATERIALS AND METHODS

Experimental Animals

Eleven of the 12 mares used in these experiments were Standardbred and one, Lilalee, was Thoroughbred (see Appendix 2 for individual description of these mares). As in Experiments 1 and 2, these mares were kept in paddocks either on Lincoln College Research Farm or the author's property, and maintained on pasture supplemented during the winter with meadow hay. For the following experiments, seasonally acyclic mares were chosen on the basis of criteria described earlier under Experiment 3; however, the test dose of oestradiol benzoate was given only to mares in Experiments 4 and 8.

Hormone Preparations

GnRH: A synthetic preparation of the hypothalamic decapeptide GnRH (LH-RH/FSH-RH) ("Lutal") was a gift from Hoechst AG, Frankfurt, West Germany. It was provided in injectable form at a concentration of 0.2 or 1.0 mg/ml.
Oestradiol: Oestradiol benzoate in oil (Intervet Laboratories Ltd, Bar Hill, Cambs) at a concentration of 5 mg/ml was used.

Progesterone: 7.5 g progesterone (4-pregnene-3,20 dione, Sigma Chemical Co.) was dissolved in 10 ml ethanol and added to 90 ml of previously heated (> 100°) peanut oil.

Experimental Protocol

Experiment 4: AIM: To study the effect of oestradiol treatment on bio- and immunoactive LH levels in serum of acyclic mares.

Three seasonally acyclic mares (Lady Sherelle, Orient Star, and Lilalee) were bled once daily for 5 days, and then received the following course of oestradiol treatment:

<table>
<thead>
<tr>
<th>Day of Experiment</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Day 1 = 1st day of sampling)</td>
<td></td>
</tr>
</tbody>
</table>
| 6 | Inject 1.5 mg oestradiol benzoate (E2B) i.m.  
Bleed at 0 and +4 h. |
| 7 | Inject 1.5 mg E2B i.m. at 0 and +8 h.  
Bleed at 0, +4 and +8 h. |
| 8 | Inject 2.0 mg E2B i.m. at 0 and +8 h.  
Bleed at 0, +4 and +8 h. |
| 9 | Bleed. |

This course of oestradiol benzoate administration was the same as that used by Vivrette and Irvine (1979) and reportedly results in serum oestradiol levels approximately 1.5 times those of the normal periovulatory surge. Mares were teased on experiment days 5 - 8, and all showed oestrous behaviour only after administration of E2B.
Experiment 5: AIM: To study the effect of progesterone treatment on bio- and immunoactive LH levels in serum of acyclic mares.

This experiment began 8 days after completion of Experiment 3, and used the same 3 mares. At the time of this experiment, Lady Sherelle and Lilalee had some follicular activity and 8 days after the end of this experiment, both were spontaneously in oestrus with pre-ovulatory sized follicles in their ovaries. Orient Star's ovaries remained inactive during this time. The three mares were bled once daily for two days and then were given 75 mg progesterone in oil i.m. twice daily at approximately 12 h intervals for two days. Daily mean serum progesterone levels induced by this treatment were \(4.7 \pm 1.1\) ng/ml (J.E. Turner, pers. comm.), which are comparable to normal dioestrous levels found using this progesterone assay (Evans and Irvine, 1975). Blood samples were taken before each progesterone injection.

Experiment 6: AIM: To study the effect of GnRH on bioactive LH in serum from cyclic mares in oestrus and dioestrus, and from seasonally acyclic mares, and to compare relative changes in bio- and immuno-active LH levels after GnRH in the three groups.

a) November 1977 - January 1978: Three mares (Prentice, True Return and Mrs Oram) were given 0.5 mg GnRH i.v. once during oestrus and once during dioestrus (see Table 7 for the exact day of the cycle on which experiments were done). Prentice and True Return were treated first in oestrus, while Mrs Oram was treated first in dioestrus. Blood samples collected at -0.5, 0, 0.5, 1, 2, and 3 h from GnRH injection were assayed for LH by \textit{in vitro} bioassay\textsuperscript{1}.

\textsuperscript{1}This experiment was part of a larger study by Foster, Evans and Irvine, the protocol and results of which have been published in J. Reprod. Fert. 56:567-572 (1979). Dr Foster performed the LH radio-immunoassays on these blood samples using the ovine-ovine system as described by Evans and Irvine (1975).
b) **May - June 1980:** Four mares (Descent, Tussle, Zany, and Safe Dream) were given 0.5 mg GnRH i.m. once during oestrus and once during dioestrus (see Table 7). Descent, Tussle and Zany were treated first in oestrus, while Safe Dream was treated first in dioestrus. Blood samples were collected from all mares at -0.75, -0.5, 0, +0.5, 1, 2 and 3 h from GnRH injection. Zany and Safe Dream were also bled at +0.25, 0.75 and 1.5 h from injection.

In both Experiments 6a and b, GnRH injections were separated by at least 8 days (see Appendix 2.)

c) **September 1980:** Three seasonally acyclic mares (Lady Sherelle, Orient Star, and Lilalee) were given 0.4 mg GnRH intramuscularly and bled at -0.3, 0, +0.5, 1, 2, and 3 h from GnRH injection. This experiment was done immediately before Experiment 4.

**Experiment 7:** AIM: To study the effect of hourly pulses of GnRH on bio- and immunoactive LH levels in acyclic mares.

Four seasonally acyclic mares (Kai Tere, Lady Sherelle, Orient Star and Prentice) were given a pulse injection of 0.25 mg GnRH intravenously hourly for 6 h and were bled at 20 min intervals from 1 h before the first GnRH injection through 2 h after the last injection. Because of the large number of blood samples to be taken, cannulae (Portex polythene tubing, PP 204, i.d. 1 mm, o.d. 1.5 mm, Portex Ltd, Hythe, Kent, England) were inserted under local anaesthetic (2 ml lignocaine hydrochloride, "Xylocaine" 2%, Astra Chemical Pty Ltd, N. Ryde, NSW, Australia) through a 14 gauge stainless steel needle into a jugular vein of each mare. The cannulae were stitched to the mare's skin and sealed between samplings by pinching over the exposed end and inserting the folded cannula into a short length of wider polythene tubing. Blood was prevented from clotting in the cannulae by filling them with 5% EDTA in
Table 7: Day of cycle on which Experiments 6a and b mares were given 0.5 mg GnRH.

<table>
<thead>
<tr>
<th>Mare</th>
<th>Experiment</th>
<th>Day of Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prentice</td>
<td>6a</td>
<td>1) Last day of oestrus.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 11th day of dioestrus.</td>
</tr>
<tr>
<td>Mrs Oram</td>
<td>6a</td>
<td>1) 10th day of dioestrus.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 2nd day of oestrus (oestrus length = 7 days).</td>
</tr>
<tr>
<td>True Return</td>
<td>6a</td>
<td>1) 2nd day of oestrus (oestrus length = 3 days).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 5th day of dioestrus.</td>
</tr>
<tr>
<td>Descent</td>
<td>6b</td>
<td>1) 3rd day of oestrus (oestrus length = 7 days).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 7th day of dioestrus.</td>
</tr>
<tr>
<td>Tussle</td>
<td>6b</td>
<td>1) 2nd day of oestrus (oestrus length = 3 days).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 7th day of dioestrus.</td>
</tr>
<tr>
<td>Safe Dream</td>
<td>6b</td>
<td>1) 7th day of dioestrus.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 2nd day of oestrus (oestrus length = 4 days).</td>
</tr>
<tr>
<td>Zany</td>
<td>6b</td>
<td>1) 2nd day of oestrus (oestrus length = 4 days).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 7th day of dioestrus.</td>
</tr>
</tbody>
</table>

1 Day 1 = first day of dioestrus.
normal saline between samplings. When withdrawing blood samples, the first 2 ml diluted with the EDTA solution was discarded.

Experiment 8: AIM: To study the effect of pretreatment with oestradiol on bio- and immunoactive LH responses to hourly GnRH pulses in acyclic mares.

Four seasonally acyclic mares (True Return, Lilalee, Bright Sea and Mrs Oram) were given 1.5 mg oestradiol benzoate intramuscularly daily for 8 days. On the last day of oestradiol treatment, the mares were given a pulse injection of 0.25 mg GnRH intravenously hourly for 3 h. Mares were bled at 2, 1, 0.5 and 0 h before the first injection and at 20 min intervals through 1.5 h after the last injection. Cannulae were not used in this experiment.

Assays

Bio- and immunoassays on samples from Experiments 5, 7, and 8 (and immunoassays on Experiment 4 and 6c samples) were made considerably later than those for Experiments 1, 2, 3 and 6a and b (1982 vs 1980-81). In the time between the two batches of assays, the LH standard appeared to have gained immunopotency (as shown by decreased potency estimates in the RIA of the replicate serum standards) resulting in elevated B:I ratios in the later experiments. Thus, to facilitate comparison of the effect of these experiments on B:I ratio, all data have been presented as per cent pretreatment B:I ratio.

Statistical Analyses

NB: Analysis of the effect of GnRH treatment on LH levels (i.e. Experiments 6, 7 and 8) will be presented in Sections 2 and 3.
Experiment 4: The effect of oestradiol on LH levels was assessed by analysis of variance, in which mean pretreatment LH levels were compared with mean levels during oestradiol treatment. The multiple blood samples within each period were considered to give replicate estimates of each mean. Individual mares were considered to be treatment replications. Bio- and immunoassay data were analysed separately. The effect of oestradiol on B:I ratio of serum LH was evaluated by analysis of variance as described above after transformation of data to log (B:I ratio).

Experiment 5: This experiment could not be analysed because LH levels in two of the mares frequently fell below the limit of detection of both bio- and immunoassays.

Experiment 6: Data from cyclic mares were pooled for analysis of variance (see Figure 16 for justification for pooling). The model used was a split plot in time, in which mares were replicates, stage of cycle (oestrus or dioestrus) was the main factor, and sequential blood samples after GnRH, subfactors (see Steel and Torrie (1980) Ch. 16.5 for discussion of the split plot in time model, and Garcia et al. (1979) for its application to analysis of treatment effects on hormone levels in sequential blood samples). In a separate analysis, data from acyclic mares were combined with those from cyclic mares for one-way analysis of variance to compare the effect of physiological state (oestrus, dioestrus, acyclic) on relative change in B:I ratio after GnRH. Treatment means (i.e., physiological states) were compared when necessary with Duncan's new multiple range test.

Experiments 7 and 8: Mean B:I ratios for periods before and between GnRH pulses were calculated for each mare from the multiple blood samples taken. These data were then expressed as log (B:I ratio) and sub-
mitted to analyses of variance in which treatment was number of GnRH pulses received (i.e., no pulse, 1 pulse, 2 pulses, etc.) and mares, treatment replications. For Experiment 8, treatment means were compared with Tukey's W test (Steel and Torrie, 1980, pg 185).

RESULTS

Experiment 4: Mean levels (+ S.E.M.) of bioactive LH in serum of three acyclic mares before, during and after oestradiol treatment are shown in Figure 13a. Mean immunoactive LH levels (+ S.E.M.) are shown in Figure 13b.

Both bio- and immunoactive LH levels were slightly but not significantly lower during oestradiol treatment than before. (BIO: no E2 = 3.17 ng/ml, E2 = 2.38 ng/ml, F1 20 = 2.98, NS. RIA: no E2 = 1.92 ng/ml, E2 = 1.45 ng/ml, F1 20 = 3.71, NS). Oestradiol did not significantly alter the ratio of bio:immunoactive LH in serum (mean post-treatment ratio = 100% pretreatment, F1 35 = 0.002, NS).

Experiment 5: Mean levels (+ S.E.M. where possible) of bioactive and immunoactive LH in serum of three acyclic mares before and during progesterone treatment are shown in Figure 14. As noted under Statistical Analyses, this experiment could not be analysed because LH levels in Lady Sherelle and Orient Star frequently fell below the limit of detection of both immuno- and bioassays. The results have been presented here, to demonstrate the unexpected surge in bio- and immunoactive LH in all three mares on the morning of the second day of progesterone treatment.

Experiment 6: Mean levels (+ S.E.M.) of bioactive LH before and after GnRH given in oestrus and dioestrus are shown for Experiment 6a and b mares in Figures 15a and 15b respectively. Mean immunoactive LH
Figure 13: LH levels in acyclic mares before, during and after treatment with oestradiol (Experiment 4).

a) Bioassay

b) Radioimmunoassay

Bars indicate the standard error of the mean.
Figure 14: Mean LH levels in acyclic mares before and during treatment with progesterone (Experiment 5).

Bars indicate the standard error of the mean which was calculated only when data were available from all 3 mares (See text).
levels for 6b mares are shown in Figure 15c. Figure 16 compares for each of the seven mares post-GnRH B:I ratio (expressed as per cent baseline ratio) in oestrus and dioestrus experiments. Mean change in B:I ratio over the three hours after GnRH injection is shown for the two cycle stages in Figure 17.

After GnRH, the change in B:I ratio was significantly greater (P < 0.001, $F^1_6 = 98.9$) in dioestrus than in oestrus, B:I ratio dropping in relation to baseline in dioestrus, but not in oestrus (oestrus mean = 104.1% baseline, dioestrus mean = 56.5% baseline, l.s.d. (P < 0.01) = 17.7). When oestrous and dioestrous experiments were grouped, there was a significant effect of time of sampling after GnRH on B:I ratio (P < 0.05, $F^3_{36} = 2.73$), with the half hour sample having a lower B:I ratio than the remaining samples. However, when the two cycle stages were considered independently; no significant effect of sampling time on B:I ratio could be demonstrated at either dioestrus or oestrus using Duncan's new multiple range test.

Mean levels (+ S.E.M.) of bio- and immunoactive LH in three acyclic mares before and after GnRH injection are shown in Figures 18a and 18b. Mean change in B:I ratio over the three hours after GnRH injection is shown in Figure 18c. When per cent change in B:I ratio induced by GnRH in these acyclic mares was compared with data from cyclic mares, a highly significant (P < 0.005) effect of the physiological state of the mare was observed, with the change in ratio after GnRH being significantly greater in dioestrous mares than either acyclic or oestrous mares. Per cent change in acyclic mares (103.2% baseline) and oestrous mares (104.1% baseline) were not significantly different.

Experiments 7 and 8: Mean levels (+ S.E.M.) of bioactive LH in three acyclic (Experiment 7) and four acyclic-estradiol-treated (Experiment 8) mares before, during and after hourly pulse injections of GnRH.

1 To avoid confusion, results of Dr Foster's immunoassay (i.e. Experiment 6a mares) have not been shown here, since his use of a different equine LH standard to CI 1-37 yielded radically different potency estimates to those presented elsewhere in this thesis.
Figure 15a: Mean LH levels (and S.E.M.) in 3 mares before and after injection of 0.5 mg GnRH in oestrus and dioestrus.
Figure 15b: Mean LH levels (and S.E.M.) in 4 mares before and after injection of 0.5 mg GnRH in oestrus and dioestrus.
Figure 15c: Mean immunoactive LH levels (and S.E.M.) in 4 mares before and after injection of 0.5 mg GnRH (at arrow) in dioestrus and oestrus.
Figure 16: Change in B:I ratio (expressed as % pre-injection value) after 0.5 mg GnRH given in oestrus and dioestrus to 7 mares.
Figure 16: cont'd

Hours from GnRH Injection

Oestrus  | Tussle  | Dioestrus  | Descent

% Baseline E/I Ratio

0  1  2  3  0  1  2  3
Figure 16: cont'd

![Graph showing changes in % baseline B:I ratio over time from GnRH injection. The graph is divided into three phases: Oestrus, ZANV, and Dioestrus. The y-axis represents the % baseline B:I ratio, ranging from 0 to 150, and the x-axis represents hours from GnRH injection, ranging from 0 to 3.]

- **Oestrus**: Shows an upward trend in % baseline B:I ratio over time.
- **ZANV**: Shows a sharp decline in % baseline B:I ratio, followed by a slight increase.
- **Dioestrus**: Maintains a relatively stable % baseline B:I ratio over time.
Figure 17. Mean B:I ratio (expressed as % baseline value) after GnRH given in oestrus and dioestrus to 7 mares.

Bars represent the standard error of the mean.
Figure 18: Mean LH levels (and S.E.M.) in 3 acyclic mares before and after injection of 0.4 mg GnRH. Ratio of bio-immunoactive LH in each serum sample is also shown.

a) Bioassay

b) Radioimmunoassay

c) B:I Ratio

Hours from GnRH Injection
are shown in Figures 19a and 20a respectively. Corresponding immunoassay data appear in Figures 19b and 20b. Data from one Experiment 7 mare (Lady Sherelle) have been omitted from analysis, since pre-GnRH levels fell below the sensitivity of the immunoassay. In Experiment 7, analysis of variance detected no significant effect of repeated GnRH injections on B:I ratio (see Figure 19c, $F_{14}^{6} = 0.438$). By contrast, analysis of Experiment 8 revealed a highly significant effect ($P < 0.001$) of GnRH pulses on B:I ratio, the ratio increasing markedly ($P < 0.01$) after the first injection and rising slightly but not significantly after the second and third injections (see Figure 20c).

NB: For all the above experiments, data from individual mares appear in Appendix 3.

DISCUSSION

The pattern of immunoactive LH levels in serum during the oestrous cycle described here agrees with that reported by others (Whitmore, Wentworth and Ginther, 1973; Geschwind et al., 1975; Evans and Irvine; 1975). Although the pattern of bioactive LH levels was qualitatively similar to this, it was not identical so that the ratio of biological:immunological LH changed significantly during the cycle; being high as LH levels rose during oestrus but falling sharply on the last day of oestrus to remain stable and low through dioestrus. Changes during the ovulatory cycle in relative in vitro biological and immunological potency of serum LH have been reported in other species having sialylated polymorphic luteinizing hormones. In an extensive study of daily blood samples from 40 cycles from 8 women, Robertson et al. (1979), using the mouse testosterone production assay (mTPA) and a homologous immunoassay, observed a small but significant depression in B:I ratio at the ovulatory LH surge. By con-
Figure 19: Mean LH levels (and S.E.M.) in 3 acyclic mares before and after hourly injections of 0.25 mg GnRH (at arrows). Mean ratio of bio- and immunoactive LH in serum before and after each injection is also shown.

(a) Bioassay

(b) Radioimmunoassay

(c) B:I Ratio

Hours
Figure 20: Mean LH levels (and S.E.M.) in 4 acyclic oestradiol-treated mares before and after hourly injections of 0.25 mg GnRH (at arrows). Mean ratio of bio:immunoactive LH in serum before and after each injection is also shown.

Bars without a common superscript are significantly different at the 0.05 level.
trast, Dufau et al. (1976a), using the rat testosterone production assay (rTPA) and anti-hCG in a homologous immuno-assay found no consistent change in B:I ratio in three human cycles. These conflicting results may be due to differences in assays used; however, they more probably stem from the inadequacy of sample size in the Dufau work. Unlike women in either report, rhesus monkey females\(^1\) were observed to have a 3′-4-fold increase in B:I ratio at the ovulatory LH surge (Dufau, Hodgen, Goodman and Catt, 1977; Neill, Dailey, Tsou and Reichert, 1977: rTPA/ovine-ovine RIA). Interestingly, recent work in which monkeys were bled intensively around the time of ovulation has shown that the bioactive LH surge begins approximately 5 h before the immunoactive surge (Marut et al., 1981; mTPA/ovine-ovine RIA). On the other hand, with radioreceptor assay (hCG-porcine granulosa cells) and the ovine-ovine RIA, a significant decrease in B:I ratio at the LH surge was found in rhesus females (Sakai and Channing, 1979). Explanations offered by all groups for the changing B:I ratio in serum LH involve speculated alterations in the nature of the circulating hormone. For the human, Robertson et al. (1979) have suggested that the lowered mid-cycle B:I ratio is due in part, to the increased secretion of gonadotrophin subunits, which has been reported to occur at this time (Hagen, McNatty and McNeilly, 1976)\(^2\) or after maximal GnRH stimulation (Hagen and McNeilly, 1975). Subunits may cross react in the radioimmunoassay depending on the quality of reagents, but are biologically inert (Suginami, Robertson and Diczfalusy, 1978). For the rhesus, it has been suggested by all workers that the altered mid-cycle...

\(^1\)Rhesus LH is presumed to be sialylated (see Peckham and Knobil, 1976a) but this has not been verified by chemical analysis.

\(^2\)Robertson et al. appear to have misinterpreted Hagen, McNatty, McNeilly's paper. Although subunit secretion DID increase during the LH surge, percent change in serum subunit levels appears to have been LESS than percent change in intact LH. If the ratio of intact LH to subunit in serum did not decline during the LH surge, the drop in B:I ratio observed cannot be attributed to an increase in degree of subunit interference in the RIA.
B:I ratio results from a structural modification of the LH molecule, probably a change in carbohydrate composition. In this case, the contradictory results may be explained by different type of assay used by Channing and Sakai (1979) to measure "biologically active" LH, since modification of the carbohydrate portion of the LH molecule may affect binding and steroidogenic potencies in opposite ways (see later, Moyle Bahl and Marz, 1975). For the horse, alterations in the carbohydrate composition of the hormone would seem the more plausible explanation of the two offered for changes in B:I ratio of serum LH, since (1) in the horse, like the monkey, ratio of steroidogenically potent to immuno-reactive LH rises with LH levels, and (2) a decreased ratio persists through dioestrus when LH secretion rate and presumably pituitary stimulation (and subunit secretion) are minimal.

Much evidence exists to suggest that the potency of gonadotrophins in various biological assays and radioimmunoassay is affected differentially by carbohydrate, particularly sialic acid, content. Because sialic acid increases the survival time of glycoproteins in plasma, possibly by reducing binding to hepatocyte membranes and consequent degradation in the liver (Morrell, Gregoriadis and Scheinberg, 1971; Pricer and Ashwell, 1971), it is not surprising that human and horse gonadotrophins lose considerable in vivo biological potency when sialic acid is enzymatically removed (Braunstein et al., 1971; Dufau, Catt and Tsuruhara, 1971; Tsuruhara, Dufau, Hickman and Catt, 1972; Schams and Papkoff, 1972; Yang and Papkoff, 1973). The desialylated hormone would have a shorter plasma half-life and duration of action than intact hormone, and therefore reduced potency in standard in vivo bio-assays. Desialylation of human LH and hCG also seems to affect in vitro biological activity, increasing potency in radioreceptor assay (rat ovary, hCG, Tsuruhara et al., 1972; rat Leydig cell, hCG, Moyle, Bahl and Marz, 1975) but decreasing steroidogenic potency (rat Leydig cells, hCG and hLH
Dufau et al., 1971; rat Leydig cell, hCG, Moyle et al., 1975; monkey granulosa cell, hCG, Channing and Bahl, 1978). Conversely, desialylation of equine gonadotrophins has been reported to enhance steroidogenic potency (rat TPA, eLH, PMSG, Aggarwal and Papkoff, 1981; rTPA, cAMP accumulation assay, PMSG, Moyle et al., 1978). Interesting parallels can be drawn here with the insulin receptor, desialylation of which has been reported to uncouple insulin binding and cell response (Jacobs and Cuatrecasas, 1976).

Finally, sialic acid appears to affect the antigenicity of glycoproteins, desialylated forms making better antigens than fully sialylated forms (Athineos, Thornton and Winzler, 1962). Thus, some antisera may recognise desialylated molecules more strongly than sialylated. Increases in radioimmunoassay potency following sialic acid removal have been observed with hFSH (Vaitukaitis and Ross, 1971), equine FSH (Aggarwal and Papkoff, 1981) and hLH (Braunstein et al., 1971), while no change in immunopotency was seen with desialylated hCG (Bahl, Marz and Moyle, 1974; van Hall et al., 1971) PMSG or equine LH (Aggarwal and Papkoff, 1981).

Summarising the above observations: removal of sialic acid from gonadotrophins decreases in vivo biological potency, has variable effects on in vitro biological potency depending on hormone and method of assay, and increases or doesn't change immunopotency. These experiments have compared extremes, i.e. fully sialylated vs enzymatically desialylated molecules. The extent to which physiological variation in sialic acid content of polymorphic gonadotrophins affects biological and immunological activities has received little attention. When equine pituitary LH is fractionated by isoelectric focussing, the ratio of in vitro biological*: immunological activity increases with decreasing isoelectric point and increasing sialic acid content (Irvine, 1979; see later,

*mTPA
Section 1, Chapter 4). Similar observations have been made on focussed hCG (Merz, Hilgenfeldt, Dorner and Brossmer, 1974).

Although neither study established that sialic acid content was the ONLY difference between molecular forms, results demonstrated that horse pituitary and human chorion: produce gonadotrophin molecules of varying biological and immunological properties, which could be related to sialic acid content.

Recent work with a number of glycoproteins has suggested that the composition of carbohydrate sidechains can be varied in response to changing physiological conditions (see, for example, reviews by Atkinson and Hakimi, 1980; Shur and Roth, 1975; Vaughan, 1982).

Interestingly, oestradiol has been shown to increase galactosyl and sialyl transferase activities in rat endometrium; this stimulatory effect being inhibited by high levels of progesterone (Nelson et al., 1975). In chick oviduct, diethylstilboestrol stimulates increased levels of dolichol phosphate, a necessary substrate of glycosyl transferases (Lucas and Levin, 1977; Burton et al., 1981). There is evidence that in several species, the steroid environment can alter certain characteristics of pituitary and serum gonadotrophins, including circulatory half-life, apparent molecular size, electrophoretic mobility and isoelectric focussing profile. A summary of relevant experiments is presented in Table 8, and it can be seen from this that the nature of the steroid effect varies with species and sex. The chemical basis for these steroid-induced alterations to gonadotrophins rarely has been investigated; one exception being a very careful study by Peckham and Knobil (1976a and b) on rhesus monkey gonadotrophins. These workers found that in both sexes gonadectomy induced a shift in gel chromatography elution profile of LH and FSH which in females (the experiment with males was not tried) could be reversed by treatment of the monkeys with oestradiol or desialylation of the castrate form of the hormone.
In vitro experiments with rat pituitaries have suggested that steroids may interact with GnRH to alter the form of secreted LH. Thus, GnRH stimulated pituitaries from intact (rat radioreceptor assay, Sharpe et al., 1975) and oestradiol-treated gonadectomised rats (but not gonadectomised rats) were found to secrete LH with a greater B:I ratio than that stored in the gland (rat radioreceptor assay, mTPA, Mukhopadhyay et al., 1979). Stored LH and LH-released without GnRH stimulation from the three types of glands had similar B:I ratios. In rat pituitary, GnRH has been reported to stimulate the incorporation of glucosamine into immunoprecipitable LH (Liu and Jackson, 1978) and it has been suggested (Todd et al., 1973; Menon et al., 1977; Liu and Jackson, 1978; Mukhopadhyay et al., 1979) that it is this GnRH stimulated attachment of carbohydrate to the previously synthesised protein core that is responsible for the altered B:I ratio of secreted LH. Furthermore, Liu and Jackson (1975) have observed that oestradiol acts synergistically with GnRH in enhancing glucosamine incorporation into LH. In vivo, GnRH stimulation has been shown to increase the B:I ratio of serum LH in cyclic women in the luteal and late but not early follicular phase. Stimulation did not alter the already elevated B:I ratios in male or post-menopausal sera (rTPA, Dufau, Beitins, MacArthur and Catt, 1976). From these results, Dufau et al. hypothesised that gonadal status and/or LH secretion rate (high in post-menopausal women and men) influenced the nature of serum LH.

The present work has shown that in the acyclic mare, neither oestradiol nor GnRH alone could alter the B:I ratio of serum LH. However, when oestradiol-pretreated acyclic mares were given hourly GnRH pulses, B:I ratio increased significantly after the first GnRH injection, and continued to rise slightly but not significantly after subsequent injections. In the cyclic mare, the B:I ratio of serum LH was found to be elevated during oestrus. Thus, it is possible that the rising serum oestradiol preceding ovulation and increased GnRH levels which may occur
at this time (Irvine, 1981; see Section 2) could cause the secretion of forms of LH with greater biological potency. GnRH given to oestrous mares failed to alter B:I ratio, but this might be expected if oestradiol and endogenous GnRH were already exerting maximal effect on the biological potency of serum LH. (Note that in E2-treated acyclic mares, B:I ratio did not increase significantly after the second and third GnRH injections.) When GnRH was given to dioestrous mares, a marked drop in B:I ratio relative to baseline values was observed, suggesting that progesterone and GnRH may interact to cause secretion of LH forms with lower biopotency. However, the lowered B:I ratio during dioestrus in the normal cycle may not be attributable solely to a depressant effect of progesterone, since B:I ratios in sera from acyclic and dioestrous mares were not significantly different. These observations suggest that the effect of progesterone on the quality of serum LH deserves further investigation.

Great individual variation in mean B:I ratio through the cycle was seen in Experiment 1. Similar observations have been made in human females (Dufau, Pock, Neubauer and Catt, 1976; Robertson et al., 1979). However, in the mare, unlike the woman (Robertson et al., 1979), cycle mean B:I ratio was significantly affected by season; being higher in late breeding season cycles than mid-season cycles. The pattern of change in B:I ratio appeared similar in mid- and late season cycles; however, the extent of change seemed to be greater mid-season than late. Endocrine events associated with the transition from ovulatory to anovulatory seasons have not been elucidated in the mare. Cycles in early and late breeding season have been reported to differ in the pattern of FSH levels (two surges/cycle in early season, one surge/cycle in late season; Turner, Garcia and Ginther, 1979). However, the relationships, if any, between this observation, ovarian steroid secretion, and the nature of serum LH remain to be established.
Finally, the physiological significance to the mare of qualitative alterations in serum LH needs clarification. The fact that the mare ovulates when relative biopotency of LH is maximal as assessed by mouse Leydig cells, suggests both that these presumed molecular changes do have physiological importance and that the response of equine target cells can be predicted adequately by the mouse. This latter observation is also supported by preliminary results from an equine Leydig cell testosterone production assay. It is noteworthy that the continuing rise in plasma LH which occurs after ovulation and is unique to the mare, seems to consist of LH of relatively low biological potency. In most species, LH measured by immuno- or bioassay is beginning to decline by the time of ovulation in accordance with the concept that the major role of LH is induction of ovulation. In the mare, it is the profile of relative biopotency of serum LH that corresponds closely to this pattern. That this late oestrus-early dioestrus change in biopotency may in itself have physiological importance is suggested by the intriguing example of the mare who had no change in ratio and who failed to maintain a corpus luteum.

The practical significance of these findings is that in the normal cycle, the radioimmunoassay system used here can provide useful measurements of LH. However, in the abnormal cycle, after GnRH or when the steroid environment is artificially altered, RIA alone may not give adequate information on the level of LH stimulation at target tissues. In these circumstances, bioassay-RIA comparisons may be necessary to detect changes in the type of circulating LH.
Table 8: Effect of steroid environment on various characteristics of pituitary and serum gonadotrophins.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weick, R.F. (1977)</td>
<td>Rat</td>
<td>The disappearance rate of serum LH after hypophysectomy is faster in intact female rats than ovariectomised.</td>
</tr>
<tr>
<td>Robertson, Foulds and Ellis (1982)</td>
<td>Rat</td>
<td>The distribution of bioactive (mTPA) pituitary LH after isoelectric focussing (IEF) is significantly altered by sex (more activity between pH 7 and 9.5 in males than females) and gonadectomy (hormone relatively more alkaline in castrates of both sexes).</td>
</tr>
<tr>
<td>Robertson, Foulds and Ellis (1982)</td>
<td>Rat</td>
<td>The distribution of bioactive pituitary FSH (calf testes RRA) after IEF is significantly affected by sex (hormone relatively more alkaline in females) but not by gonadectomy.</td>
</tr>
<tr>
<td>Solano, Garcia-Vela, Catt and Dufau (1980)</td>
<td>Rat</td>
<td>B:I ratio (rTPA) of serum and pituitary LH in male rats declines transiently after castration, with return to pre-castration value by 60 d post-operation.</td>
</tr>
<tr>
<td>Bogdanove, E.M., Nolin, J.M. and Campbell, G.T. (review, 1975)</td>
<td>Rat</td>
<td>Pituitary and serum FSH in intact males have a greater B:I ratio (Steelman-Pohley assay), larger apparent molecular size on gel filtration and a longer plasma half-life than FSH from castrates. The effect of castration on FSH is reversed by testosterone treatment. Pituitary and serum FSH in intact females have a lower B:I ratio, and smaller apparent molecular size than FSH from castrated males.</td>
</tr>
<tr>
<td>Strollo et al. (1981)</td>
<td>Human</td>
<td>The distribution of bioactive (mTPA) serum LH after IEF is significantly different in postmenopausal women than cyclic women at the ovulatory LH surge (hormone relatively more alkaline in cyclic women).</td>
</tr>
<tr>
<td>Reference</td>
<td>Animal</td>
<td>Observations</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Robertson, van Damme and Diczfalusy (1977)</td>
<td>Human</td>
<td>The distribution of bioactive (mTPA) pituitary LH after IEF is significantly different in post-menopausal women than cyclic women at the LH surge (hormone relatively more alkaline in cyclic women).</td>
</tr>
<tr>
<td>Peckham, Yamaji, Dierschke and Knobil (1973)</td>
<td>Rhesus monkey</td>
<td>Serum and pituitary FSH from intact female rhesus monkeys have 1) a lower B:I ratio (Steelman Pohley assay); 2) smaller apparent molecular size on gel filtration; and 3) shorter plasma half-life than serum and pituitary FSH from ovariectomised females.</td>
</tr>
<tr>
<td>Peckham and Knobil (1976a and b)</td>
<td>Rhesus monkey</td>
<td>Pituitary LH from intact males and females has a smaller apparent molecular size on gel filtration and a shorter plasma half-life than LH from gonadectomised animals. In females, effect of ovariectomy on LH is reversed by oestradiol treatment, or removal of sialic acid from castrate form of the hormone.</td>
</tr>
</tbody>
</table>
CHAPTER IV.

ISOELECTRIC FOCUSING OF HORSE PITUITARIES AND SERA
The preceding experiments provided strong evidence for the occurrence of qualitative changes in serum LH during the equine oestrous cycle. The following experiments sought to define more precisely the nature and source of these changes, firstly, by studying the effect of physiological state of the pituitary donor on LH distribution after isoelectric focussing and secondly, by comparing the isoelectric focussing profile of serum LH (of high and low B:I ratio) with that of pituitary LH. An important feature of these experiments was the fractionation of individual pituitaries and sera. In all other studies using isoelectric focussing to examine the polymorphism of gonadotrophins, pools of pituitaries or sera have been used. Thus, it is not outside the realm of possibility that the molecular "heterogeneity" observed was the sum of variations (possibly genetically directed) between individuals in the type of molecule produced.

Isoelectric Focussing: A Definition

"Perhaps the most ingenious and effective electrophoretic method for separating proteins is isoelectric focussing or electro-focussing, invented by H. Svensson in Sweden in which the mixture of proteins is subjected to an electric field in a gel support in which a pH gradient has first been generated. Each protein then migrates toward, and is "focused" at, that portion of the pH gradient where the pH is equal to its isoelectric pH and forms a sharp stationary band there." (Lehninger, 1975)

1With the exception of Robertson et al. (1977), who did include data for a single post-menopausal pituitary. However, the isoelectric focussing procedure used to fractionate this pituitary was considered to be sub-optimal, resulting in damage to the focussed LH.
MATERIALS AND METHODS

Description and Preparation of Samples for Isoelectric Focussing

1. Pituitaries: Five individual pituitary homogenates were fractionated by isoelectric focussing. Information about the horses from which the pituitaries were taken is given in Table 9. All pituitaries were collected within 15 min of the horse's death, and placed immediately on ice.

Table 9: Description of horses from which pituitaries were collected for isoelectric focussing.

<table>
<thead>
<tr>
<th>Pituitary Code</th>
<th>Horse</th>
<th>Date of Collection</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Horse mare, 6 years old</td>
<td>2/7/80</td>
<td>Large preovulatory follicle found in ovary on autopsy.</td>
</tr>
<tr>
<td>B</td>
<td>&quot;Wild Mist&quot;, horse mare, 16 years old</td>
<td>1/10/80</td>
<td>In oestrus when killed. Large preovulatory follicle found in ovary on autopsy.</td>
</tr>
<tr>
<td>C</td>
<td>&quot;Michigan&quot;, horse mare, 26 years old</td>
<td>1/10/80</td>
<td>&quot;Post-menopausal&quot;¹, given 2.5 mg oestradiol benzoate i.m. daily for 5 days before death.</td>
</tr>
<tr>
<td>D</td>
<td>Pony mare, aged</td>
<td>2/7/80</td>
<td>Probably &quot;post-menopausal&quot;, inactive ovaries.</td>
</tr>
<tr>
<td>E</td>
<td>Horse gelding, 3 years old</td>
<td>3/7/80</td>
<td></td>
</tr>
</tbody>
</table>

¹i.e., anovulatory due to age.
In the laboratory, the pituitaries were weighed and prepared for isoelectric focussing as described by Robertson et al. (1977). Pituitaries A and B were bisected after weighing and half of each pituitary was also extracted using the method of Braselton and McShan (1970). For the Robertson method, each gland was homogenised at 4°C in 2-5 volumes of 0.25 M sucrose - 0.14 M NaCl in distilled water, using a ground glass homogeniser. The homogenate was centrifuged at 103,000 x g for 90 min at 4°C. The supernatant was removed and dialysed (Visking, 36/32, Kempthorne Prosser, Christchurch) overnight at 4°C against distilled water, and then for a further 16 h against 50% sucrose in water. This second dialysis served to concentrate the sample, which was then stored at -20°C until isoelectric focussing. For the Braselton and McShan method, each half pituitary was homogenised in 3 volumes of 0.025 M PO₄ buffer, pH 7.3 and then extracted twice with 40% ethanol (1 g pituitary/3 ml ethanol). The precipitate resulting from this step was removed by centrifugation at 770 x g for 10 min at 4°C, and the supernatants from the two ethanol extractions pooled, and cooled to -15°C. Five volumes of ice cold acetone were then added. The resulting precipitate was removed by centrifugation as described above, washed with 2.5 volumes of acetone, dried and taken up in 0.05 M NaCl. This preparation was dialysed overnight at 4°C against 0.025% NaCl and the precipitate which formed during dialysis removed by centrifugation. The supernatant was concentrated by dialysis overnight against 50% sucrose and then lyophilised. Finally, the dried supernatant was reconstituted in 0.025 M PO₄ buffer, pH 7.3, to an approximate concentration of 2 g dialysate/100 ml buffer, placed in an ice bath and constantly stirred while the pH was lowered to 4.2 with 0.035 M phosphoric acid. The resulting precipitate was removed by centrifugation and the pH of the supernatant adjusted to 7.0 - 7.2 with 10 N NaOH. This supernatant was concentrated by dialysis overnight against 50% sucrose, and then stored at -20°C until isoelectric focussing.

1Except pituitary E which was stored frozen whole from 2/7/80 to 1/10/80.
2. Sera: Four serum samples were fractionated by isoelectric focussing. Two mares (Shorett and Fourth Hall, see Appendix 2) were bled once daily through a normal oestrous cycle. Serum LH levels were measured by bio- and immuno-assay in those samples collected several days before the onset of oestrus through the first few days of dioestrus; and these data and the ratio of bio- to immunoactive LH in each sample are shown in Figure 21.

For each mare, the serum sample with the highest B:I ratio was selected (in both mares, this sample was collected on the last day of oestrus) as well as samples with considerably lower B:I ratios collected during early dioestrus (see Figure 21; mean B:I ratio in the two "high ratio" samples chosen for isoelectric focussing = 1.35; in the three "low ratio" samples = 0.77). To concentrate the sera for isoelectric focussing, 2 ml were lyophilised in glass scintillation vials, and reconstituted in 0.5 ml distilled water.

Isoelectric Focussing Procedure

Isoelectric focussing (IEF) was performed in a flat-bed of granulated gel following LKB Application Note 198 with a few modifications and using the LKB-Multiphor system and accessories for isoelectric focussing (LKB Produkter AB, Bromma, Sweden). Ampholyte solution (5.5 ml, 'Ampholine', pH range 3.5 - 10, LKB Produkter AB) was mixed with 104.5 ml distilled water. Five g Sephadex G-75 superfine (for IEF, Pharmacia, Uppsala, Sweden) was added to 100 ml ampholyte solution to form a slurry which was degassed under vacuum. Eight electrode strips were cut (1 x 7 cm, Whatman paper #17), six of which were soaked in the remaining 10 ml of diluted ampholyte. Three strips were placed at each narrow end of an 11 x 24 cm glass plate which was then weighed. The gel slurry was poured onto the plate, which was weighed again, and the weight of added gel calculated. Water was evaporated from the gel under: a) a stream of $N_2$ (pituitaries), or b) warm air (sera), to
Figure 21: Bio- and immunoactive LH levels and the ratio between the 2 activities in serum samples collected around the time of oestrus from the mare, Fourth Hall. Samples selected for isoelectric focussing are indicated by arrows.
Figure 21: cont'd Mare = Shorett

Day of Cycle

1 = first day of dioestrus.
bar indicates period of behavioural oestrus.
approximately 3/4 of the gel crack point (see Application Note 198 for calculation). This took approximately 3 h after which the plate was placed on the cooling unit of the isoelectric focussing system. The efficiency of cooling was increased by: 1) coating the top of the unit with a thin layer of detergent or kerosene to aid conduction; and 2) slowly pumping the tap water supply to the unit through a large bath of ice. One of the remaining two electrode strips was soaked in 1 M phosphoric acid, the other in 1 M NaOH, and these were placed on the ampholyte strips at either end of the plate. The gel was then prefocussed at 8 W (350 V x 24 mA) for 30 min to remove possible contaminants in gel or ampholytes (Vaughan, 1982). Samples (150 - 250 µl) were applied to 1.5 x 4.0 cm filter paper strips (Whatman #17) which were gently placed on the gel approximately 0.75 cm from the cathodal end, and at least 0.5 cm from the side of the plate. Four samples were run on each gel plate, and thus three isoelectric focussing runs were made; the first containing pituitaries A and E extracted using "Robertson" or "Braselton and McShan" methods, the second, pituitaries B, C and D, and the third, sena. To encourage elution of the samples from the strips, the system was run at 2 W (200 V x 10 mA) for 2 h, after which the strips were removed and the power was increased to 4 W (400 V x 10 mA) and the system run overnight. In the morning (~4 h later), current had usually declined to 5 mA and because the power supply was set to deliver a constant voltage (i.e., 400 V), power output to the plate was 2 W. This was increased to 8 W (1000 V x 8 mA) for 2 h to sharpen the definition of focussed bands. Total focussing time was between 18 and 20 h. The gel was then divided perpendicularly to the long axis of the plate into 30 x 0.5 cm sections using a stainless steel fractionating grid, and into 4 columns parallel to the long axis of the plate, following the line of sample application. These gel sections were scooped from the plate (which was left on the cooling unit), placed in either: a) 2 ml syringe barrels the outlets of which were covered with approximately 0.25 cm of wet,
packed glass wool (pituitary experiments), or b) polypropylene elution columns, the outlets of which were covered with 10 μm mesh nylon net (LKB 2117-502, LKB Produkter, Sweden) (serum experiments), and eluted with 2 ml distilled water into 10 ml glass test tubes. Care was taken to keep the eluted fractions cold by packing the test tubes in ice and by frequent transfer to a refrigerator. When all gel sections had been eluted, the pH gradient was measured in one vertical column of fractions (pH meter = Corning EEL digital 112). The uniformity of the gradient across the plate was confirmed by measuring the pH of selected fractions from other vertical columns. Fractions were then dialysed (Visking 36/32) overnight at 4°C against running tap water to remove ampholytes, poured into Wasserman tubes, capped and stored at -20°C until assay.

The results of the isoelectric focussing procedure were reproducible as indicated by the similarity in pI values of haemoglobin (identified as several sharp red bands in the gel) in the two pituitary focussing runs; being 7.10 in Run 1 and 7.02 in Run 2 (haemoglobin wasn't present in the serum run). Furthermore, the pI's of LH peaks were extremely consistent between focussing runs (see later).

1. Recovery of LH after IEF:

   a) Pituitaries: Mean recovery of focussed pituitary LH was 54.0 ± 7.7% (X ± S.E.M., recovery being calculated for each sample by summing the amount of LH measured in each fraction after focussing and dividing this total by the amount of LH in the sample loaded onto the plate). Recovery appeared to vary with IEF run, being better in the second run (pituitaries B, C and D, mean recovery ± S.E.M. = 77.8 ± 7.3%) than the first run (pituitaries A, A "Braselton and McShan" and E; mean recovery ± S.E.M. = 38.5 ± 5.7%) (see Table 10). Bio- and immunoassay recoveries were similar in the four pituitaries for which the comparison was available (BTO = 45.8 ± 9.0%, RIA = 48.8 ± 13.5%). However, results may have been
<table>
<thead>
<tr>
<th>Pituitary Code</th>
<th>Wet Weight Tissue (g)</th>
<th>Extracted From Pituitary (µg)</th>
<th>Focussed (µg)</th>
<th>Recovered (µg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BIO</td>
<td>RIA</td>
<td>BIO</td>
<td>RIA</td>
</tr>
<tr>
<td>A</td>
<td>0.9</td>
<td>659.0</td>
<td>417.3</td>
<td>98.8</td>
<td>62.6</td>
</tr>
<tr>
<td>B</td>
<td>2.2</td>
<td>-</td>
<td>621.1</td>
<td>-</td>
<td>93.2</td>
</tr>
<tr>
<td>C</td>
<td>2.4</td>
<td>548.0</td>
<td>960.1</td>
<td>82.2</td>
<td>144.0</td>
</tr>
<tr>
<td>D</td>
<td>1.7</td>
<td>-</td>
<td>9.2</td>
<td>-</td>
<td>12.8</td>
</tr>
<tr>
<td>E</td>
<td>1.0</td>
<td>64.0</td>
<td>66.5</td>
<td>9.6</td>
<td>10.0</td>
</tr>
<tr>
<td>A*</td>
<td>1.1</td>
<td>75.3</td>
<td>156.8</td>
<td>11.3</td>
<td>23.5</td>
</tr>
</tbody>
</table>

*Extracted using the Braselton and McShan method.
affected by inter-assay variation; the majority of bio- and immunoassays of fractions being done between July 1980 (bio- and immunoassays of pituitary A) and March, 1982 (radioimmunoassays pituitaries B, C and D), while assays of unfraccionated pituitaries and serum concentrates were made in September 1982.

b) Sera: Mean recovery of focussed serum LH was $85.0 \pm 15.2\%$ ($X \pm S.E.M.$); however, results varied with method of assay and serum sample (see Table II). Recovery of immunoactive LH exceeded bioactive ($110.3 \pm 20.7$ vs $59.8 \pm 15.0\%$); recovery of bioactive LH being especially poor from the two high B:I ratio samples (high B:I $\approx 39.5\%$ vs low B:I $\approx 80.0\%$). This will be discussed in detail later.

Assays

LH content of all fractions was determined by radioimmunoassay as described earlier, except that serum was not added to standards or blanks. The immunoassay design used for all pituitary fractions was 2-point dilutions (ratio dilution 1:dilution 2 = 2) of fractions in duplicate. LH content of fractions from isoelectric focussing of sera and of pituitaries A, B and D was also measured by in vitro bioassay. The assay design used was either 2-point dilutions (ratio dilution 1:dilution 2 = 2) of fractions in triplicate (radioimmunoassays and bioassays of serum fractions) or 3-point dilutions (ratio of dilutions 1:2:3 = 3) of fractions in triplicate (bioassays of pituitary fractions).

Figures 22 and 23 compare in a representative radioimmunoassay and bioassay, dilutions of fractions after isoelectric focussing of pituitary A with CI 1-37 standard curves. In both assays, the majority of fraction dilutions were visually parallel to the standard curve; paired t-test

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1 In a most unfortunate accident, fractions from pituitaries C and E were left out of the freezer at least overnight, before bioactive LH content had been determined. It was decided not to bioassay these fractions because loss of bioactive LH caused by the accident would have made results difficult to interpret.
Table 11: Recovery of LH after isoelectric focusing of serum.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Focussed (ng)</th>
<th>Recovered (ng)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BIO</td>
<td>RIA</td>
<td>BIO</td>
</tr>
<tr>
<td>Fourth Hall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high B:I ratio</td>
<td>23.8</td>
<td>16.0</td>
<td>9.9</td>
</tr>
<tr>
<td>low B:I ratio</td>
<td>15.1</td>
<td>19.5</td>
<td>15.6</td>
</tr>
<tr>
<td>Shorett</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high B:I ratio</td>
<td>33.7</td>
<td>28.7</td>
<td>12.6</td>
</tr>
<tr>
<td>low B:I ratio</td>
<td>20.2</td>
<td>26.2</td>
<td>11.6</td>
</tr>
</tbody>
</table>
Figure 22: Comparison of dilutions of the equine LH standard (CI 1-37) and fractions after isoelectric focussing of Pituitary A: in vitro bioassay.
Figure 23: Comparison of dilutions of the equine LH standards (CI 1-37), and fractions after isoelectric focusing of pituitary A: radioimmunoassay.
indicating no reason to assume non-parallelism (RIA; \( t = 0.94, \text{df} = 23, \) NS, BIO; \( t = -0.22, \text{df} = 19, \) NS).

Peak Definition

Peaks were subjectively defined by inspection of graphed data, and are marked on appropriate figures. Only fractions at the top of each peak or shoulder were used to calculate pI values and B:I ratios. When bio- and immunoassay disagreed on the shape of a peak, calculations were based on bioassay values for pituitary fractions and immunoassay values for serum fractions (due to problems with the bioassay in such fractions; see later).

RESULTS

Pituitaries

Figures 24 - 28 show, for each pituitary, immunoactive and, where possible, bioactive LH profiles after isoelectric focussing. In all pituitaries, the bulk of LH activity was found in the pH region 4.5 - 7.5, in which 3 - 4 peaks of activity were apparent. In the three pituitaries in which bioactive LH was measured, bio- and immunoactive LH peaks concurred. Mean pI values (+ S.D.) for peaks were: 7.19 ± 0.29 (n = 4), 6.06 ± 0.04 (n = 4), 5.18 ± 0.01 (n = 5) and 4.53 ± 0.11 (n = 5). Table 12 shows, for each pituitary, the percentage of LH activity occurring in the various pH ranges. The gelding pituitary was unique in that 80.9% of bioactive LH and 71.9% of immunoactive LH were found between pH 5.0 and 5.9, while only 11.5% of bioactive LH and 17.0% of immunoactive LH occurred below pH 5.0. In the four mare pituitaries, the distribution of activity between these two regions was approximately equal. The proportion of bio- or immunoactivity found above pH 6.0 was small in all pituitaries (maximum =
Figure 24: LH levels in fractions after isoelectric focussing of oestrous mare pituitary A.
Legend for Figures 24 - 28, 30 - 31 and 33 - 34.

Bio- and/or immunoactive LH levels in fractions after isoelectric focussing of horse pituitaries or serum. The pH of each fraction is also shown. Horizontal bars indicate peaks of LH activity (See text), vertical bars indicate fractions containing haemoglobin (Hb).
Figure 25: LH levels in fractions after isoelectric focusing of oestrous mare pituitary B.

Fraction lost during dialysis.
Figure 26. LH levels in fractions after isoelectric focusing of oestradiol-treated aged mare pituitary C.

Bioassay limit of detection = 0.04 μg/fraction.
Figure 27: LH levels in fractions after isoelectric focusing of aged mare pituitary D.
Figure 28: LH levels in fractions after isoelectric focussing of gelding pituitary E.

Bioassay limit of detection fractions 1-9 = 0.02 μg/fraction.
22%, pituitary A) and in several instances, distinct peaks of activity were difficult to define in the region. For example, the peak with a pI value of 7.19 clearly existed only in oestrous mare pituitary A and aged mare pituitary D; while the pI 6.06 peak occurred as just a small shoulder of the major peak at pI 5.18 in the gelding and aged mare pituitaries. Figure 29 shows the ratio of biological:immunological LH activity in fractions after isoelectric focussing of pituitaries A, C and E. The mean B:I ratio in peak fractions is shown in Table 13.

B:I ratios of isoelectric focussing fractions ranged from: 0.62 - 1.59 in the oestrous mare pituitary, 0 (immunological activity but no detectable bioactivity; bioassay limits of detection are indicated where relevant on Figures 26 and 28) to 2.95 in the gelding pituitary and 0 - 0.75 in the E₂-treated aged mare pituitary. For the three sets of fractions, geometric mean B:I ratio was greatest at the LH peak with a pI value of 5.18 and lowest at the pI 7.19 peak (see Table 13). It should be noted that peak fractions from the E₂-treated pituitary C did not show this pattern of B:I ratio; the ratio being greatest at the pI 7.19 peak and declining with pH.

Bio- and immunoactive LH profiles after isoelectric focussing of "Braselton and McShan" extracts of pituitaries A and E are shown in Figures 30 and 31. As with "Robertson" extracts, the bulk of LH activity was found in 3 - 4 peaks in the pH region 4.5 - 7.5 with similar distribution of bio- and immunoactive LH. Mean pI values for peaks agreed well with those after "Robertson" extraction and were: 7.58, 6.0, 5.29 and 4.59. However, the percentage of LH activity found in the various pH regions differed markedly between extraction methods, with a mean of 22.5% of bio-active and 20.8% of immunoactive LH occurring above pH 7.0 in "Braselton and McShan" extracts (see Table 14). In "Robertson" extracts, only 2.1% of bioactive and 3.4% of immunoactive LH were found in this region.
Table 12: The distribution of bio- and immunoactive LH after isoelectric focussing of 5 pituitaries, expressed in terms of percentage LH activity recovered within various pH ranges.

<table>
<thead>
<tr>
<th>Pituitary</th>
<th>pH Range</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0-4.9</td>
<td>5.0-5.9</td>
<td>6.0-6.9</td>
<td>&gt; 7.0</td>
</tr>
<tr>
<td>A Oestrous mare</td>
<td>BIO</td>
<td>27.2</td>
<td>52.4</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>28.5</td>
<td>49.4</td>
<td>16.9</td>
</tr>
<tr>
<td>B Oestrous mare</td>
<td>RIA</td>
<td>50.7</td>
<td>43.4</td>
<td>4.4</td>
</tr>
<tr>
<td>C E₂-treated mare</td>
<td>BIO</td>
<td>37.4</td>
<td>54.6</td>
<td>5.9</td>
</tr>
<tr>
<td>D Aged mare</td>
<td>RIA</td>
<td>41.3</td>
<td>50.3</td>
<td>5.2</td>
</tr>
<tr>
<td>E Gelding</td>
<td>BIO</td>
<td>11.5</td>
<td>80.9</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>17.0</td>
<td>71.9</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Table 13: Ratio of biological:immunological LH activity in peak fractions after isoelectro-focussing of three pituitaries.

<table>
<thead>
<tr>
<th>Pituitary</th>
<th>PI Value of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.19</td>
</tr>
<tr>
<td>A</td>
<td>0.91</td>
</tr>
<tr>
<td>C</td>
<td>0.57</td>
</tr>
<tr>
<td>E</td>
<td>0*</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>0.37</td>
</tr>
</tbody>
</table>

* Arbitrarily assigned a value of 0.1 to calculate geometric mean.
Figure 29: Ratio of biological:immunological activity in fractions after isoelectric focussing of 3 individual pituitaries.

Pituitary C
E2 Treated Mare

Pituitary A
Oestrous Mare

Pituitary E
Gelding

pH
Figure 30: LH levels in fractions after isoelectric focussing of gelding pituitary E extracted using the method of Braselton and McShan.

Bioassay limit of detection fractions 11-24 = 0.05 μg fraction.
Figure 31: LH levels in fractions after isoelectric focusing of pituitary A extracted using the method of Braselton and McShan.
Table 14: The distribution of bio- and immunoactive LH after isoelectric focussing of two pituitaries extracted using the Braselton and McShan method; expressed in terms of percentage LH activity recovered within various pH ranges.

<table>
<thead>
<tr>
<th>Pituitary</th>
<th>pH Range</th>
<th>4.0-4.9</th>
<th>5.0-5.9</th>
<th>6.0-6.9</th>
<th>≥ 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BIO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Oestrous mare</td>
<td>BIO</td>
<td>8.5</td>
<td>62.1</td>
<td>13.2</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>11.4</td>
<td>59.6</td>
<td>12.9</td>
<td>16.2</td>
</tr>
<tr>
<td>E Gelding</td>
<td>BIO</td>
<td>38.1</td>
<td>28.1</td>
<td>4.9</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>12.3</td>
<td>47.1</td>
<td>14.9</td>
<td>25.4</td>
</tr>
</tbody>
</table>

Figure 32 shows the B:I ratio in fractions after isoelectric focussing of "Braselton and McShan" extracts, and Table 15 shows the mean B:I ratio in peak fractions. B:I ratios of fractions ranged from: 0 - 0.95 in the oestrous mare pituitary, and 0 - 5.71 in the gelding pituitary. For the two sets of fractions, geometric mean B:I ratio was lowest at the LH peak with a pI value of 5.29 in marked contrast to fractions from "Robertson" extracted pituitaries in which B:I ratio was greatest at this peak. In Braselton and McShan extracted pituitaries, mean B:I ratio was greatest at the pI 4.59 peak. This was due solely to a relative increase in the gelding pituitary in B:I ratio in fractions below pH 5.0 when compared to more alkaline fractions (see Figure 32). In the Braselton and McShan extracted pituitary, the B:I ratio of LH in each of the four peaks was similar, differing too from the Robertson extraction in which a distinct peak in B:I ratio occurred at pI 5.18.
Figure 32: Ratio of biological:immunological activity in fractions after isoelectric focussing of 2 pituitaries extracted using the method of Braselton and McShan.
Table 15: Ratio of biological immunological LH activity in peak fractions after isoelectric focussing of two pituitaries extracted using the method of Braselton and McShan.

<table>
<thead>
<tr>
<th>Pituitary</th>
<th>pI Value of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.58</td>
</tr>
<tr>
<td>A Oestrous mare</td>
<td>0.78</td>
</tr>
<tr>
<td>E Gelding</td>
<td>1.35</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>1.03</td>
</tr>
</tbody>
</table>

**Sera**

Figures 33 and 34 show, for each of the four serum samples, immunoactive and bioactive LH profiles after isoelectric focussing. In all sera, the bulk of LH activity was found in the pH region 4.0 - 7.5, in which 2 - 4 peaks of activity were apparent. Unlike pituitaries after IEF, bio- and immunoactive peaks did not always concur, especially at either extreme of the pH range. Mean pI values (+ S.D.) for concurrent bio- and immunoactive peaks were 5.74 + 0.14 (n = 4), 5.10 + 0.08, (n = 4), 4.57 + 0.08 (n = 4) and 4.05 (n = 1). These values are similar to those observed in focussed pituitaries (i.e., 6.06, 5.12 and 4.53). Immunoactive only peaks occurred at pI values of 4.15 + 0.08 (n = 2), 6.43 (n = 2) and 7.32 (n = 3). Table 16 shows for each serum sample the percentage of LH activity occurring in the various pH ranges.

Unlike focussed pituitaries, no bioactivity was found above pH 6.0, whereas the percentage of immunoactive LH in this region was similar in pituitaries and sera. No consistent difference between high and low B:I ratio sera in distribution of LH activity could be observed. In Fourth Hall, the per cent immunoactive LH occurring above pH 6.0 in the low B:I
Figure 33: LH levels in fractions after isoelectric focusing of 2 serum samples from the mare, Fourth Hall; one sample having a high B:I ratio, one having a low B:I ratio.

Filled in bar indicates fraction containing substance that interfered in the bioassay.
Figure 34: LH levels in fractions after isoelectric focusing of 2 serum samples from the mare, Shorett, one sample having a high B:I ratio, one having a low B:I ratio.

Filled in bar indicates fraction containing substance that interfered in the bioassay.
ratio serum was more than twice that in the high ratio sample (15% vs 7%), while distribution of both bio- and immunoactive LH below pH 6.0 was similar in high and low ratio sera. On the other hand, in Shorett, the percentage of bio- and immunoactive LH found below pH 5.0 was increased in the low B:I ratio sample (BIO:25% vs 13%, RIA:32% vs 19%) with a concomitant decline in per cent activity in the pH range of the major peak (i.e., pH 5.0 - 5.9).

Table 16: The distribution of bio- and immunoactive LH after isoelectric focussing of four serum samples; expressed in terms of per-cent age LH activity recovered within various pH ranges.

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>4.0-4.9</th>
<th>5.0-5.9</th>
<th>6.0-6.9</th>
<th>&gt; 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth Hall</td>
<td>BIO</td>
<td>RIA</td>
<td>BIO</td>
<td>RIA</td>
</tr>
<tr>
<td>High B:I</td>
<td>45</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low B:I</td>
<td>48</td>
<td>52</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Shorett</td>
<td>BIO</td>
<td>RIA</td>
<td>BIO</td>
<td>RIA</td>
</tr>
<tr>
<td>High B:I</td>
<td>13</td>
<td>87</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low B:I</td>
<td>25</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 17 shows the mean B:I ratio in peak fractions from each focussed serum sample. Figure 35 shows the B:I ratio in fractions after IEF of the four sera. The range of B:I ratios was similar in all focussed sera and extended from 0 (immunoactivity but no detectable bioactivity) to 1.13 (excepting 3 fractions of Fourth Hall's high B:I ratio sample which contained bioactivity with no detectable immunoactivity and for which a B:I ratio could not be calculated). For the four sets of fractions, arithmetic mean B:I ratio was greatest at the LH peak with a pI value of 5.12, declining on either side of this peak to low values at acidic and alkaline extremes. This pattern of B:I ratio is similar to that observed in focussed pituitaries (see Table 13).

Table 17: Ratio of biological:immunological LH activity in peak fractions after isoelectric focussing of four serum samples.

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>pI Value of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.12</td>
</tr>
<tr>
<td>Fourth Hall</td>
<td></td>
</tr>
<tr>
<td>High B:I</td>
<td>-</td>
</tr>
<tr>
<td>Low B:I</td>
<td>0.68</td>
</tr>
<tr>
<td>Shorett</td>
<td></td>
</tr>
<tr>
<td>High B:I</td>
<td>0</td>
</tr>
<tr>
<td>Low B:I</td>
<td>0</td>
</tr>
<tr>
<td>Mean*</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Arithmetic - due to frequency of occurrence of 0 values.
Figure 35: Ratio of biological:immunological activity in fractions after isoelectric focussing of 4 serum samples from two horses.

Whole serum had a high B:I ratio.

Whole serum had a low B:I ratio.

* Samples with bioactivity but no detectable immunoactivity.
Although results of serum IEF appeared to agree well with those of pituitary IEF, interpretation of the serum experiment was complicated by the discovery of a substance that interfered in the bioassay in several fractions of three of the focussed sera. This substance was first detected in fractions in the pH range 4.7 - 5.3 from both of Shorett's serum samples. In the bioassay, but not the immunoassay, dilutions of these fractions were not parallel to the standard curve, in fact the smaller the amount assayed, the greater appeared the LH content. In several fractions, the greatest amount added (8.3 μl) reduced cell testosterone production below basal level (no added LH). Only one fraction (pH 5.12) from Fourth Hall's high B:I ratio sample and no fractions in her low B:I ratio sample showed similar interference in the bioassay. Bioassay interference was not observed in fractions from focussed pituitaries. Several experiments were then done to identify the distribution and mode of action of the interfering substance in Shorett's fractions and, if possible, to reduce its effect.

Potency estimates given previously were derived from least amount of added fraction.

1) Selected fractions from Shorett's high and low B:I ratio sera were assayed with 100, 200 or 400 pg standard LH. 10 μl of each fraction was used, which was slightly greater than the maximum amount previously assayed (8.3 μl). Shorett's high B:I ratio serum was reconstituted by pooling fractions, and the pool assayed at several dilutions to test recovery of focussed LH and parallelism to the standard curve. The unfocussed serum was also assayed at two dilutions.

2) Ten μl of the pH 4.95 fraction from Shorett's high B:I ratio sample (fraction #G17 - which was in the middle of the region of maximum interference as judged from Experiment 1) was assayed with:
   a) 10 μl low LH serum and 0 or 400 pg standard LH,
   b) 2 μl oestrous mare serum,
   c) 1 μl Shorett high B:I ratio serum
   d) 20 ng* progesterone and 0 or 100 pg LH
3) Fraction #G17 was heated to 70°/30 min and 10 μl assayed with 0 or 100 pg LH.
4) Ten μl of fraction #G17 was added to cells at 34° 30 min after 0 or 100 pg LH.
5) Cells (approximately 10^6 in 200 μl TCM) were incubated for 2 h at 34° with or without 20 μl of the pH 4.95 fraction from Shorett's low B:I ratio sample. Cell viability after incubation was assessed by trypan blue exclusion.

*Equivalent to 2.5 μg/ml serum at maximum amount of serum added to the bioassay.
Parallelism to the standard curve was impossible to test because of inadequate LH concentrations; however, a "reverse" dose-response relationship seen earlier in pH 4.7 - 5.3 fractions was not observed with the pool. The unfocussed serum was acceptably parallel to the standard curve (i.e., the difference between potency estimates derived from the two dilutions < 20%). Addition of various sera with fraction G#17 did not reduce its suppressive effect, nor did addition of excess progesterone (see Table 19).

Table 19: Percent suppression of testosterone production by fraction #G17 in presence of various sera or excess progesterone.

<table>
<thead>
<tr>
<th>% Suppression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>G17 alone</td>
<td>64</td>
</tr>
<tr>
<td>+ 10 µl low LH serum</td>
<td>55</td>
</tr>
<tr>
<td>+ 10 µl low LH serum + 400 pg LH</td>
<td>57</td>
</tr>
<tr>
<td>+ 2 µl oestrous mare serum</td>
<td>72</td>
</tr>
<tr>
<td>+ 1 µl Shoret high B:I ratio serum</td>
<td>76</td>
</tr>
<tr>
<td>+ 20 ng progesterone</td>
<td>67</td>
</tr>
<tr>
<td>+ 20 ng progesterone + 100 pg LH</td>
<td>62</td>
</tr>
</tbody>
</table>

At this high level of added progesterone, cell testosterone production was similar to 0 and 100 pg standard LH and exceeded maximum LH stimulated production in the absence of progesterone (412 pg testosterone at 100 pg LH vs 1216 pg at 100 pg LH + 20 ng progesterone). Nevertheless, fraction G#17 could reduce this "progesterone-stimulated" testosterone production by over 60%. Heating fraction G#17 to 70°/30 min virtually eliminated its bioassay interference (% suppression of testosterone production: heated G#17 alone = 0%, heated G#17 + 100 pg LH = 19%). This experiment
also ruled out the possibility that the substance affected binding of 
testosterone to testosterone antibody since in all bioassays, assay 
tubes are heated to 72 - 75\degree C/30 min before testosterone radioimmunoassay. 
As a further test, 10 \mu l of unheated G\#17 was added to "zero added 
testosterone" tubes in the testosterone RIA. Per cent counts bound was 
similar in the presence and absence of the fraction (62% bound with G\#17 
vs 59% bound without G\#17). Adding G\#17 to cells, at 34\degree C, 30 min after 
0 or 100 pg LH, did not reduce appreciably bioassay interference (% 
suppression - G\#17 alone = 43\%, G\#17 + 100 pg LH = 59\%). After 30 min 
at 34\degree C, LH binding to Leydig cell receptors has approached maximum (Chen 
et al., 1979) and testosterone secretion stimulated by a maximal LH dose 
has significantly exceeded basal secretion (Garfink et al., 1976). Cell 
viability assessed by trypan blue exclusion was similar after 2 h incubat-
ion with and without the pH 4.95 fraction from Shorett's low B:I ratio 
sample.

From these experiments, it could be concluded that the interfering 
substance in Shorett's high B:I ratio sample:

1) Was distributed in a broad band between pH 4.15 and 5.5
2) Could not be detected in unfocussed serum or focussed serum 
   reconstituted by pooling fractions.
3) Was not created by IEF per se since no interference was 
   observed in fractions from Fourth Hall's low ratio serum.
4) Was heat labile, and therefore possibly a protein.
5) Could not be counteracted by addition with serum.
6) Appeared to act either along the synthetic pathway between 
   progesterone and testosterone or on the secretion of 
   testosterone into incubation medium (cells are not lysed 
   by heating to 72\degree C, followed by freezing/thawing).
Results of these experiments indicated that per cent suppression of LH stimulated testosterone production by various fractions from Shorett's focussed serum was greatest between pH 4.77 and 5.50 and lowest (<15%) at either pH extreme (see Table 18). When pH 4.95 fractions from Shorett's high and low ratio sera were compared in the same assay, testosterone production was suppressed slightly more by the high ratio fraction (56% vs 49%). Recovery of focussed LH from pooled fractions was 55% (18.5 ng recovered); however, the LH concentration in the pool was at the limit of assay detection, and difficult to measure accurately.

Table 18: Per cent suppression\(^1\) of the LH stimulated testosterone production by fractions after IEF of serum\(^2\).

<table>
<thead>
<tr>
<th>Serum</th>
<th>pH of Fraction</th>
<th>LH Added (pg)</th>
<th>% Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shorett</td>
<td>8.00</td>
<td>100</td>
<td>11</td>
</tr>
<tr>
<td>high B:I ratio</td>
<td>7.32</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6.43</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5.12</td>
<td>200</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>4.96</td>
<td>400</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>4.77</td>
<td>100</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>4.15</td>
<td>100</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>Shorett</td>
<td>4.96</td>
<td>400</td>
<td>49</td>
</tr>
<tr>
<td>low B:I ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) % suppression = \[
\left(1 - \frac{\text{testosterone produced in presence of fraction}}{\text{testosterone produced in absence of fraction}}\right) \times 100
\]

\(^2\) Data have been collected from three separate assays.
The interfering substance seemed to be more effective in high B:I ratio sera than low ratio sera. This was certainly the case with Fourth Hall, and may have been true too for Shorett, judging from recovery of focussed bioactive LH from the two sera (high ratio, 37% recovery vs low ratio, 57% recovery). Thus, if the substance were active in unfocussed serum, the difference between high and low B:I ratio sera would be underestimated. In focussed serum, failure to inactivate the substance makes interpretation of results, particularly Shorett's, difficult. However, because of the similarity between patterns of focussed LH activity in Shorett's samples (high interference), Fourth Hall's samples (little - no interference) and pituitaries (no interference) it has been assumed that in Shorett the major effect of the interfering substance was to reduce the amount of bioactive LH measured between pH 4.15 and 5.5 but not its pattern of distribution.

DISCUSSION

Polymorphism of highly purified equine pituitary LH has been reported after isoelectric focussing in several studies with peaks of activity occurring at pH 7.3, 6.6, 5.9 and 4.8 - 4.5 (Braselton and McShan, 1970) or 9.0, 7.3, 6.2, with a broad peak between 5.1 and 4.1 (inspection of data, Reichert, 1971). Irvine (1979), focussing a crude ethanol extract of horse pituitaries (the "Braselton and McShan extraction method" used in the present work) and assaying fractions in the pH range 4.0 - 7.0, observed a broad multiphasic peak of LH activity between pH 5.1 and 4.3\(^1\). In the present work, isoelectric focussing (IEF) of crude aqueous extracts of horse pituitary resulted in LH peaks at pH 7.2, 6.1, 5.2 and 4.5; values reasonably comparable to those obtained in the other.

\(^1\)To enable comparison between Irvine's study and the present one, these pH values have been adjusted to correct for difference between studies in the observed isoelectric point of the internal marker, haemoglobin (7.06, this study vs 6.8, Irvine, pers. comm.).
studies, although the existence in purified preparations of a distinct LH peak at pH 5.1 - 5.2 may be questionable. However, this could be explained by selective loss (or discard) of these molecules during rigorous purification. For example, the preparation focussed by Braselton and McShan contained only 10% of the LH activity in the initial crude pituitary extract. Furthermore, compared with earlier work, LH peaks in the present study were more sharply defined, which could be attributed to the use of flat bed rather than column IEF. In flat bed IEF, diffusion of focussed bands can be prevented during fraction collection by imbedding a stainless steel grid in the gel (LKB Application Note, 198); no such control can be used during column elution. Similarities between this and earlier studies in pI values of LH peaks were not necessarily expected, since in the present work, single pituitaries were focussed rather than pools. Therefore, these results demonstrate that within individual pituitaries, several different forms of the LH molecule exist. Furthermore, it appears that individual pituitaries differ very little in the forms of LH they are capable of producing (thus the similarities in all studies in pI values of LH peaks).

Assuming equal extraction efficiency, pituitary immunoactive LH content was vastly greater in oestrous and oestradiol-treated mares than in the "post-menopausal" mare (382.3 vs 5.4 µg/g gland extracted by RIA); however, distribution of LH activity after isoelectric focussing was similar in the four pituitaries. By contrast, focussed hormone from gelding pituitary seemed to be relatively more alkaline than that from mare pituitaries. Whether this observation reflects real differences between gelding and mare pituitary LH requires further study. The patterns of biological and immunological LH activity after IEF in each of the 3 pituitaries assayed by both methods were similar, but not identical so that the B:I ratio of LH peaks differed, mean ratio being greatest (1.01) in the pI 5.2 peak and least (0.37) in the 7.2 peak. These observations are
consistent with earlier work (Irvine, 1979) in which pI-dependent variations in B:I ratio of focussed eLH were also observed, the ratio increasing steadily between pH 6.5 (0.87) and 4.2 (4.64). Irvine focussed a pool of pituitaries collected mostly from geldings, and extracted by the Braselton and McShan method (1970). In the present study, less efficient extraction of pituitary LH (see Table 10) and, after IEF, an altered distribution of bio- and immunoactivity were observed with the Braselton and and McShan method compared with milder aqueous extraction. Interestingly, results with Braselton and McShan extracted gelding pituitary (see Figures 28 and 32) were more similar to those reported by Irvine (1979).

After IEF of human pituitary LH, no consistent variation in B:I ratio was noticed in the pH range 7.0 - 9.0; however, B:I ratio significantly decreased below pH 7.0, suggesting to the authors the presence in this acidic region of subunits, immunologically cross-reacting gonadotrophins or biologically inactive LH populations (Robertson and Diczfalusy, 1977). In the rat, the B:I ratio of focussed pituitary LH was found to vary with pH, being significantly lower in castrates and proestrous females between pH 7.0 and 9.5 than elsewhere. Conversely, B:I ratio did not vary with pH in focussed pituitaries from intact males and dioestrous females (Robertson et al., 1982). In this study, bio- (mTPA), and immunoactive rat LH were observed in all animals to be distributed in two broad peaks of activity between pH 3 and 11, contrasting markedly with the tidy peak of bioactive LH between pH 8.6 and 9.3 reported for the rat by Reichert (1971). Possibly, methods of assay or extraction have contributed to the disagreement between workers; however, until an explanation for the discrepancies is offered, results of Robertson's study should be accepted with caution.

Because the pituitary undoubtedly contains LH molecules at various stages of synthesis, the physiological importance of finding forms in the pituitary with different B:I ratios could be challenged. However,
when horse serum was focussed, marked similarities between serum and pituitary LH were observed, with peaks of LH activity occurring at like pH values, and B:I ratio changing similarly with the pH of peaks. Serum LH was different to pituitary LH in that immunoactivity without bioactivity was found at either pH extreme. Comparison of isoelectric focussing profiles of high and low B:I ratio sera (paired samples from two mares) revealed no consistent shift in LH distribution with decreased ratio; however, in both low ratio sera percent LH activity increased in areas of low B:I ratio (i.e., acid and alkaline extremes) with a concomitant drop in activity in the highest ratio peak. In the human, the IEF profiles of post-menopausal sera and pituitaries were similar, with bioactive peaks (RIA not done) occurring at like pH values (Robertson et al., 1977). However, significant differences after IEF were observed between sera from post-menopausal and cyclic (at LH surge) women, LH being relatively more alkaline in cyclic women. It was suggested that the endocrine differences between the two groups of women had resulted in structural differences in the LH molecule (Strollo et al., 1981).

While interpretation of the results of the present work is complicated by several factors, i.e. the small number of pituitaries and sera focussed, and the presence in serum from one mare of a substance that interfered in the bioassay, several observations may be cautiously made: 1) serum and pituitary horse LH are polymorphic on isoelectric focussing; 2) the different forms of the hormone appear to behave differently in bio- and immunoassay; and 3) changes in B:I ratio in whole serum seem to be mirrored by subtle changes in LH distribution after IEF. Taken together, these observations imply that the changes during the normal cycle in the B:I ratio of serum LH may indeed arise from structural changes in the circulating molecule and not from assay artefact. Whether the polymorphism of serum LH originates entirely from the pituitary or at least partially from post-secretory modification of the molecule (Campbell et al.,
1978) cannot be answered from these experiments. The close correspondence between the pI values at which the greatest part of pituitary and serum LH occurred attest to the pituitary being the major source of the polymorphism of serum LH. However, the presence in serum, but not pituitary, of LH with little or no bioactivity (and the increased distribution of LH in these forms in low ratio sera), suggest that some post-secretory modifications may occur.
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hormone, ovine follicle-stimulating hormone, pregnant mare's
serum gonadotropin and human chorionic gonadotropin in the ham-
SECTION 2.

INVESTIGATIONS OF MECHANISMS REGULATING LH LEVELS IN CYCLIC MARES
CHAPTER I.

"PITUITARY RESPONSIVENESS": LH RESPONSES TO EXOGENOUS GnRH AT VARIOUS STAGES OF THE EQUINE OESTROUS CYCLE.
Because of potential use in fertility control, mechanisms regulating serum LH levels during the ovulatory cycle have been subject to intensive research in many species. It seems evident from this body of work that the cyclic pattern of serum LH levels results from complex interplay between hypothalamic gonadotrophin releasing hormone and gonadal steroids, with the relative importance of steroidally mediated changes in pituitary responsiveness to GnRH stimulation and patterns of GnRH secretion in producing the LH surge remaining controversial. Because of the long duration of her ovulatory surge, the mare could be extremely useful for investigating factors controlling the LH surge. Thus, the following experiments were designed to determine the contribution to the mare's LH surge of changing pituitary responsiveness to GnRH. To this end, small pulse injections of GnRH were given to mares at various times during the ovulatory cycle, and pituitary responsiveness (measured as induced LH release/unit GnRH given; Yen et al, 1975) compared with pre-injection LH levels. It was reasoned that these two parameters would be highly correlated when pituitary responsiveness was the primary determinant of serum LH levels (see for example Yen et al, 1975; Lincoln, 1978; Santen and Ruby, 1979).
MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

23 of the 24 mares used in these experiments were Standardbred, and 1, Lilalee, was Thoroughbred (See Appendix 2). Maintenance of these mares has been described under Materials and Methods, Section 1. Methods used to determine oestrus or, for experiment 12, seasonal acyclicity were also discussed in Section 1.

B. HORMONE PREPARATIONS

GnRH: A synthetic preparation of the hypothalamic decapetide GnRH (LH-RH/FSH-RH) was a gift from Hoechst AG, West Germany. It was provided in injectionable form at a concentration of 0.2 or 1.0 mg/ml.

Oestradiol: 0.33g of oestradiol 17β (Sigma Chemical Co.) was dissolved in 10ml ethanol and added to 90ml previously heated (100°) peanut oil. Alternatively, oestradiol benzoate (Intervet Laboratories Ltd, Cambs) 5mg/ml in oil, was used.

Prostaglandin: Synthetic prostaglandin analogue (cloprostenol, Estrumate®, ICI New Zealand Ltd, Wellington) was used at a concentration of 250μg/ml citrate buffer.

C. CHOICE OF GnRH DOSE

The GnRH doses used in these experiments (ie. 0.32 or 0.5mg/400-500kg horse) were chosen on the basis of earlier experiments in which different GnRH doses had been given to acyclic mares (Evan's thesis, 1977). In these experiments, a dose of 0.5mg
was found to induce measurable but not maximal LH release (See also Results, Experiment 11).

EXPERIMENTAL PROTOCOL

Experiment 9

Aim: To compare GnRH induced LH release in oestrus and dioestrus. Details of this experiment are given under Materials and Methods, Section 1, Experiment 6. Briefly, 7 mares were given 0.5mg GnRH once during oestrus and once during mid-dioestrus, blood collected at frequent intervals before and after injection and serum LH measured by in vitro bioassay and radio-immuno-assay.

Experiment 10

Aim: To study the responses to GnRH given once daily throughout oestrus and to compare these responses with that induced by a single GnRH injection in mid-dioestrus.

Two mares (Coming In and Lady Sherelle) were given 0.32mg GnRH i.m. once daily through oestrus and again on the sixth day of dioestrus. Blood was collected at -0.67, -0.33, 0, 0.5, 1.0, 1.5 and 3.0h from GnRH injection, and LH levels measured by radio-immuno-assay.

Experiment 11

Aim: To study the responses to GnRH given once daily throughout an oestrous cycle.

Six mares were given 0.5mg GnRH i.m. once daily through an oestrous cycle beginning either on the first day of oestrus (Prentice, Mrs Oram and Oriental Scott) or the first day of dioestrus (Stoat, Zany, and Orient Star). Two mares (Mrs Oram and Oriental Scott) failed to return to oestrus during the treated cycle and in these mares GnRH administration was terminated after 44 and 32 days, respectively. Blood was collected from all mares
at 0, 1 and 2h from GnRH injection and LH levels measured by
*in vitro* bioassay in all (Stoot, Zany and Prentice) or selected
(Orient Star, Oriental Scott and Mrs Oram) samples. This
experiment was part of a larger study performed by Dr. Margaret
Evans, Prof. C.H.G. Irvine and Miss Julie Turner (1980).

CALCULATION OF LH RESPONSE TO GnRH INJECTION

Experiments 9 and 10

The LH response to GnRH was expressed as area units and
was calculated using the following formula:

\[
\text{RESPONSE} = (\text{area under graph of serum LH levels after GnRH
injection}) - (\text{area under graph of serum LH levels before GnRH
injection}).
\]

This calculation assumed that GnRH administration would
not affect directly or indirectly endogenous GnRH secretion which
would continue to maintain pre-injection LH levels during the
exogenously induced LH response. Although there is no proof of the
validity of this assumption in the horse, the small amount of
evidence available from other species would suggest it may be
reasonable. For example, episodic LH if not suppressed in women
by GnRH infusions which generate marked LH increases (Wang, Lasley,
Lein and Yen, 1976) or in ovariectomised sheep (Coppings and Malven,
1975) or in monkeys (Knobil, 1974) by LH or hCG infusion. The area
under the graph of LH levels was estimated as shown in Figure 36.
Response was measured as area in preference to peak change in LH
levels (See for example Yen et al, 1975) since area estimates did
not depend on accurate assay of a single sample (ie. the "peak")
and could take into account varying LH response shapes.

Experiment 11

Because of the infrequency of blood sampling in this
experiment, the area of the LH response could not be confidently
Figure 36. Calculation of area under the LH response curve

\[
\text{width} = \text{constant } k = 1 \text{ sampling interval}
\]

\[
\text{area} = \text{height} \times \text{width}
\]

Pre-injection area over 1 h = \[\frac{a + 2b + c}{2}\] x \[\frac{1}{2}\] i.e. 2 sampling intervals were summed to get total area

Post-injection area = \[\frac{c + 2d + 2e + 2f + g}{2}\] x \[\frac{1}{2}\] i.e. 4 sampling intervals were summed to get total area

*When sampling intervals were unequal, units were expressed per shortest sampling interval.*
calculated. Therefore, response was expressed as peak change in LH levels after GnRH injection.

STATISTICAL ANALYSES

Experiment 9

For all mares, oestrous and dioestrous pre-injection LH levels measured by bioassay were compared by analysis of variance, in which stage of cycle was treatment and individual mares, replicates. Similar analysis was made of oestrous and dioestrous GnRH induced bio-active LH responses. For experiment 9b mares (=6b mares, See Section 1), bio- and immuno- active LH responses were also compared using analysis of variance. The data from these mares were selected for this analysis because both bio- and immuno- assays were performed by the author, using the same LH standard, and therefore results would be more comparable than those from the bioassay and Dr. Foster's assays (ie. Experiment 9a=6a, see Section 1). For all mares the ratio between LH response and pre-injection LH levels (ie. fractional change in LH levels induced by GnRH) was calculated for oestrous and dioestrous experiments, and values for this ratio at the two cycle stages compared by analysis of variance as described above. For Experiment 9b mares, time to peak bio- and immuno- active LH levels after GnRH was also determined and the effect of cycle stage and method of assay on peak time tested by analysis of variance. Because Experiment 9a mares were given GnRH by a different route of administration, the effect of cycle stage on time to bioactive LH peak was analysed separately in this group.

Experiment 10

This experiment was not analysed because of the small number of mares sampled (n=2).
Experiment 11

Comparisons between mean 1) oestrous and dioestrous pre-injection baselines, 2) oestrous and dioestrous peak change in LH levels and 3) oestrous and dioestrous fractional change in LH levels, were made for all mares by paired t-test. In the 3 mares for which all samples had been bio-assayed, pre-injection LH levels and LH response (i.e. peak change in LH levels in this experiment) were also expressed as percent respective maximum value in each mare (eg. \[ \text{LH response day 11, mare 1} \times 100 \] )

These 3 cycles were then normalised to the first day of dioestrus and the first day of oestrus, and mean pre-injection LH levels and LH response calculated for each day of the cycle. The correlation between these two parameters during the oestrous cycle was determined by regression analysis and calculation of the correlation coefficient, \( r \) (Statistical Program Pack, HP41CV handheld calculator).

RESULTS

Mean serum levels (\( \pm \text{S.E.M.} \)) of bio- and immuno-active LH before and after 0.5mg GnRH given in oestrus and dioestrus are shown for Experiment 9b mares in Figures 15b and c. Bio-active LH levels in Experiment 9a mares are shown in Figure 15a. Table 20 shows pre-injection bio-assayable LH levels, area of the GnRH-induced response and fractional change in LH levels for all mares at both cycle stages. Table 21 shows comparable immuno-assayable LH levels for Experiment 9b mares. As expected, pre-injection LH levels were significantly higher in oestrus than mid-dioestrus (\( \bar{X} \pm \text{S.E.M.}, \) oestrus: 67.0\( \pm \) 18.2 ng/ml; dioestrus: 24.0\( \pm \) 8.1ng/ml; \( F_9^{16} = 6.95, p < 0.05 \)).
TABLE 20: Pre-injection bio-active LH Levels, area of LH response and fractional change in LH levels induced by 0.5mg GnRH given to 7 mares once during oestrus and once during dioestrus.

<table>
<thead>
<tr>
<th>MARE</th>
<th>Pre-injection [LH] (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrus</td>
<td>Dioestrus</td>
<td>Oestrus</td>
</tr>
<tr>
<td>Descent</td>
<td>28.6</td>
<td>3.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Tussle</td>
<td>27.3</td>
<td>10.9</td>
<td>21.4</td>
</tr>
<tr>
<td>Safe Dream</td>
<td>65.5</td>
<td>53.8</td>
<td>31.5</td>
</tr>
<tr>
<td>Zany</td>
<td>23.7</td>
<td>7.7</td>
<td>16.1</td>
</tr>
<tr>
<td>Prentice</td>
<td>155.5</td>
<td>21.2</td>
<td>201.4</td>
</tr>
<tr>
<td>Mrs Oram</td>
<td>66.5</td>
<td>15.6</td>
<td>67.7</td>
</tr>
<tr>
<td>True Return</td>
<td>102.0</td>
<td>55.0</td>
<td>43.0</td>
</tr>
<tr>
<td>MEAN</td>
<td>67.0</td>
<td>24.0</td>
<td>55.7</td>
</tr>
<tr>
<td>SEM</td>
<td>18.2</td>
<td>8.1</td>
<td>25.4</td>
</tr>
</tbody>
</table>
TABLE 21: Pre-injection immuno-active LH levels, area of LH response and fractional change in LH levels induced by 0.5mg GnRH given to 4 mares once during oestrus and once during dioestrus.

<table>
<thead>
<tr>
<th>MARE</th>
<th>Pre-injection [LH] (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrus</td>
<td>Dioestrus</td>
<td>Oestrus</td>
</tr>
<tr>
<td>Descent</td>
<td>20.6</td>
<td>1.7</td>
<td>9.2</td>
</tr>
<tr>
<td>Tussle</td>
<td>19.6</td>
<td>8.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Safe Dream</td>
<td>30.1</td>
<td>20.4</td>
<td>16.1</td>
</tr>
<tr>
<td>Zany</td>
<td>33.8</td>
<td>9.3</td>
<td>8.6</td>
</tr>
<tr>
<td>MEAN</td>
<td>26.0</td>
<td>9.9</td>
<td>11.0</td>
</tr>
<tr>
<td>SEM</td>
<td>3.5</td>
<td>3.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>
For the group, stage of cycle did not affect the area of the bio-active response induced by 0.5mg GnRH ($\bar{X} \pm $ S.E.M. oestrus = 55.7 ± 25.4 units, dioestrus = 57.0 ± 12.6 units, $F_6^1 = 0.003$, NS).

However for Experiment 9b mares, a significantly greater response was induced by GnRH in dioestrus than oestrus, regardless of the method of assay used ($\bar{X} \pm $ S.E.M., oestrus: bio-assay = 19.5 ± 4.7 units, RIA = 11.0 ± 1.7 units, dioestrus: bio-assay = 61.6 ± 17.3 units, RIA = 60.2 ± 11.3 units, effect of cycle stage, $F_3^1 = 20.3$, $p < 0.025$, effect of assay, $F_6^1 = 0.41$, NS). Two of the 3 experiment 9a mares were treated later in dioestrus than the rest of the group (day 10,11 versus day 5,7) and this may explain the lower dioestrus response observation in Experiment 9a. (See also results Experiment 11).

For all mares the fractional change in bio-active LH levels after GnRH was greater in dioestrus than oestrus ($\bar{X} \pm $ S.E.M., oestrus: 0.71 ± 0.13, dioestrus: 4.1 ± 1.3, $F_6^1 = 6.97$, $p < 0.05$). This was also true when Experiment 9b mares were considered separately, and method of assay did not affect results ($F_6^1 = 2.22$, NS). In Experiment 9b mares time to the LH peak was longer in oestrus than dioestrus, again regardless of method of assay used ($\bar{X} \pm $ S.E.M., oestrus: bio-assay = 116min ± 28min, RIA = 94 ± 30 min, dioestrus: bio-assay = 60min ± 21min, RIA = 56 ± 18min, effect of cycle stage, $F_3^1 = 18.9$, $p < 0.025$, effect of assay, $F_6^1 = 0.37$, NS). No significant difference in time to bio-active LH peak could be detected between mares given GnRH intravenously (See Experiment 9a) or intramuscularly (See Experiment 9b) ($\bar{X} \pm $ S.E.M., oestrus: I.V. = 100 ± 40min, dioestrus: I.V. = 50 ± 10min) and when the groups were pooled the effect of cycle stage on bio-active peak time remained significant (group mean ± S.E.M. oestrus = 109 ± 21min, dioestrus = 56 ± 12min, $F_{12}^1 = 4.75$, $p < 0.05$).

Experiment 10 and 11

Table 22 shows pre-injection immuno-assayable LH levels, area of the induced LH response, and fractional change in LH levels.
TABLE 22: Pre-injection immuno-active LH levels, area of LH response and fractional change in LH levels induced by 0.32mg GnRH given to 2 mares once daily through oestrus and on the sixth day of dioestrus.

<table>
<thead>
<tr>
<th>Date and Sexual Receptivity*</th>
<th>Pre-injection LH' (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MARE - Coming In</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21/12 +</td>
<td>32.3</td>
<td>4.6</td>
<td>0.14</td>
</tr>
<tr>
<td>22/12 +</td>
<td>39.0</td>
<td>1.9</td>
<td>0.09</td>
</tr>
<tr>
<td>23/12 o</td>
<td>54.6</td>
<td>40.5</td>
<td>0.74</td>
</tr>
<tr>
<td>28/12 -</td>
<td>8.4</td>
<td>20.2</td>
<td>2.40</td>
</tr>
<tr>
<td><strong>MARE - Lady Shere11e</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24/12 +</td>
<td>11.7</td>
<td>3.2</td>
<td>0.27</td>
</tr>
<tr>
<td>26/12 +</td>
<td>33.8</td>
<td>11.9</td>
<td>0.35</td>
</tr>
<tr>
<td>27/12 +</td>
<td>59.4</td>
<td>25.8</td>
<td>0.43</td>
</tr>
<tr>
<td>28/12 +</td>
<td>72.5</td>
<td>30.5</td>
<td>0.42</td>
</tr>
<tr>
<td>29/12 o</td>
<td>54.1</td>
<td>38.4</td>
<td>0.71</td>
</tr>
<tr>
<td>30/12 -</td>
<td>34.5</td>
<td>10.9</td>
<td>0.32</td>
</tr>
<tr>
<td>6/1 -</td>
<td>3.4</td>
<td>21.9</td>
<td>6.44</td>
</tr>
</tbody>
</table>

* +=receptive, o=passive-indifferent, -=antagonistic
in 2 mares given 0.32 mg GnRH once daily through oestrus and on the sixth day of dioestrus. In both mares, the fractional change in LH levels after GnRH was always less than one during oestrus (Coming In, $\bar{X}=0.31$, Lady Sherelle, $\bar{X}=0.42$) but had increased to 6.44 (Coming In) and 2.40 (Lady Sherelle) on the sixth day of dioestrus.

Table 23 shows pre-injection bio-assayable LH levels and peak and fractional changes in LH levels in 6 mares given 0.5 mg GnRH once daily through an oestrous cycle. Dioestrous and oestrous means ($\pm$ S.E.M.) for each parameter in each mare are also given in Table 23.

Pre-injection LH levels were greater in oestrus than dioestrus ($p<0.02$), however peak change in LH levels induced by GnRH was greater in dioestrus than oestrus ($p<0.05$) resulting in the fractional change in LH levels after GnRH being much greater in dioestrus than oestrus ($p<0.005$). Daily mean pre-injection LH levels and response to GnRH (data expressed as percentage of maximum value) in the 3 mares for which all samples were bio-assayed are shown in Figure 37. In all mares, pre-injection LH levels formed a pattern identical to that of a normal oestrous cycle. In each mare, peak GnRH induced change in LH levels was greatest during the first 5 days of dioestrus, falling gradually to a stable low baseline (Group mean $\pm$ S.E.M.: 9.2 $\pm$ 0.6% of maximum response) which was maintained until late oestrus when response again increased. For the group, pre-injection LH levels were positively correlated with response to GnRH ($r=0.68$), however the correlation was much higher during early and mid-dioestrus (dioestrus - days 1-16, $r=0.932$) than during late dioestrus - early oestrus (last 3 days of dioestrus, first 3 days of oestrus, $r=-0.06$) when pre-injection LH levels began to rise in the ovulatory surge unaccompanied by an increase in LH response to exogenous GnRH. (See Figure 37)

The results of Experiments 9-11 will be discussed with those of the following experiments at the end of this Section.
Table 23. Pre-injection bioactive LH levels, and peak and fractional changes in LH levels induced by 0.5 mg GnRH given once daily to 6 mares through an oestrous cycle

<table>
<thead>
<tr>
<th>mare</th>
<th>STOAT</th>
<th>ZANY</th>
<th>ORIENT STAR</th>
<th>PRENTICE</th>
<th>O.SCOTT</th>
<th>MRS ORAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of Exp.</td>
<td>Sexual Recaptivity</td>
<td>Pre</td>
<td>Peak</td>
<td>Frac</td>
<td>Pre</td>
<td>Peak</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>33.3</td>
<td>14.2</td>
<td>0.43</td>
<td>-</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>32.0</td>
<td>47.0</td>
<td>1.5</td>
<td>-</td>
<td>13.9</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>17.2</td>
<td>32.8</td>
<td>1.9</td>
<td>-</td>
<td>9.2</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>7.6</td>
<td>15.3</td>
<td>2.1</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>5.8</td>
<td>14.9</td>
<td>2.6</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>3.7</td>
<td>3.1</td>
<td>0.84</td>
<td>-</td>
<td>3.2</td>
</tr>
<tr>
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<td>-</td>
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<td>5.3</td>
<td>2.9</td>
<td>-</td>
<td>6.4</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>3.3</td>
<td>2.3</td>
<td>0.70</td>
<td>-</td>
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<tr>
<td>9</td>
<td>-</td>
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<td>5.2</td>
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<td>-</td>
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<td>3.8</td>
<td>3.2</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>2.2</td>
<td>3.1</td>
<td>1.4</td>
<td>-</td>
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</tr>
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<td>1.3</td>
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</tr>
<tr>
<td>13</td>
<td>-</td>
<td>1.5</td>
<td>1.3</td>
<td>0.87</td>
<td>-</td>
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<td>14</td>
<td>-</td>
<td>0.6</td>
<td>3.1</td>
<td>5.2</td>
<td>-</td>
<td>0.9</td>
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<td>15</td>
<td>-</td>
<td>0.7</td>
<td>1.3</td>
<td>1.8</td>
<td>-</td>
<td>1.7</td>
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<tr>
<td>16</td>
<td>+</td>
<td>0.8</td>
<td>1.6</td>
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<tr>
<td>17</td>
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<td>18</td>
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<td>20</td>
<td>+</td>
<td>5.8</td>
<td>2.3</td>
<td>0.40</td>
<td>+</td>
<td>6.4</td>
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<td>21</td>
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<td>0.46</td>
<td>+</td>
<td>11.0</td>
</tr>
<tr>
<td>22</td>
<td>+</td>
<td>5.8</td>
<td>3.9</td>
<td>0.67</td>
<td>+</td>
<td>15.5</td>
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<td>0.57</td>
<td>+</td>
<td>16.3</td>
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<tr>
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<td>11.9</td>
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<td>-</td>
<td>+</td>
<td>40.2</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>10.0</td>
<td>13.0</td>
<td>1.3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>18.5</td>
<td>6.5</td>
<td>0.35</td>
<td>+</td>
<td>MARE DIDN'T RETURN TO OESTRUS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tr>
<td>DX 6.3</td>
<td>8.5</td>
<td>1.9</td>
<td>24 0.08</td>
<td>7.0</td>
<td>1.4</td>
<td>20 0.08</td>
<td>17.8</td>
<td>2.1</td>
</tr>
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<td>0.38</td>
<td>2.0</td>
<td>0.17</td>
<td>0.47</td>
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<td>0.26</td>
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<td>DX 8.1</td>
<td>4.8</td>
<td>0.54</td>
<td>0.58</td>
<td>10.9</td>
<td>4.0</td>
<td>0.32</td>
<td>30.4</td>
<td>5.6</td>
</tr>
<tr>
<td>SE 1.0</td>
<td>1.6</td>
<td>0.15</td>
<td>0.23</td>
<td>2.3</td>
<td>1.8</td>
<td>0.09</td>
<td>4.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>

MARE DIDN'T RETURN TO OESTRUS
Figure 37. Mean pre-injection LH levels and LH response to GnRH (both expressed as % respective maximum value) in 3 mares given 0.5 mg GnRH once daily throughout an anoestrous cycle.

\[ \text{LH response} \]
\[ \text{pre-injection LH levels} \]

Cycles were normalised to the first day of dioestrus and the first day of oestrus. The solid horizontal bar indicates the duration of oestrus in each mare; arrows mark the day of ovulation for each mare (Z=Zany, S=Stoat, P=Prentice). Vertical bars denote the standard error of each mean (not shown unless the mean comprised data from all 3 mares). Also shown is the correlation coefficient, \( r \), between those values for LH response and pre-injection LH levels indicated between the thin vertical lines.
CHAPTER II.

CAN "PITUITARY RESPONSIVENESS" BE MEASURED IN CYCLIC MARES? INVESTIGATION OF GnRH DOSE- LH RESPONSE RELATIONSHIPS IN CYCLIC AND SEASONALLY ACYCLIC MARES.
In Experiments 9 and 10 it was observed that the responsiveness of the mare's pituitary to GnRH was lower in early oestrus than in early dioestrus (days 5-7), whereas pre-injection LH levels were higher in oestrus than dioestrus. Experiment 11 confirmed these results and demonstrated that the ovulatory LH surge began without a corresponding change in pituitary responsiveness. If the onset of the surge could not be attributed to increased pituitary responsiveness to GnRH, then it seemed most likely to be caused by increased GnRH stimulation of the pituitary. Following this reasoning, Prof. CHG Irvine proposed (1980) that data from these experiments could be used to quantitate changes in endogenous GnRH activity. Basically, Irvine theorised that if the size of the LH response induced by GnRH injection depended on the GnRH dose given and the responsiveness of the pituitary to stimulation then, similarly, LH response to ENDOGENOUS GnRH (i.e. pre-injection LH levels) would depend on GnRH input to the pituitary and pituitary responsiveness to GnRH. Thus, assuming pituitary responsiveness was the same to endogenous and exogenous GnRH, endogenous GnRH activity could be deduced by comparing pre-injection LH levels with the LH response induced by a small dose of exogenous GnRH. This somewhat difficult concept is illustrated diagrammatically in Figure 38. Fundamental to Irvine's theory was the assumption that the GnRH dose - LH response relationship was one of direct proportionality in the region of endogenous and small exogenous GnRH doses, so that in this region LH release/unit GnRH (pituitary responsiveness) would be constant. Preliminary dose - response
**Figure 38. Calculation of Irvine's GnRH Index**

\[
\text{LH release/unit GnRH} = \frac{\text{endogenous GnRH input (units)}}{\text{LH release/unit GnRH}}
\]

\[
\text{LH response} = \frac{\text{GnRH dose (units) X LH release/units GnRH}}{\text{LH release/unit GnRH}}
\]

\[
\text{pre-injection LH levels} = \frac{\text{endogenous GnRH input (units) X LH release/unit GnRH}}{\text{exogenous GnRH dose X LH release/unit GnRH}}
\]

\[
\text{LH release/unit GnRH} \text{ is 'pituitary responsiveness'.}
\]

\[
\text{pre-injection LH levels} = \frac{\text{LH response}}{\text{endogenous GnRH input (units) X LH release/unit GnRH}}
\]

\[
\text{LH release/unit GnRH} = \frac{\text{exogenous GnRH dose X LH release/unit GnRH}}{\text{LH response}}
\]

\[
\text{pre-injection LH levels} \times \text{GnRH dose} = \text{GnRH index}
\]
experiments with acyclic mares and survey of reports of similar
experiments in other species suggested that the GnRH dose - LH
response relationship could be described by a rectangular hyperbola,
which in the lower range of doses was indistinguishable from a
linear dose - linear response relationship (See later for further
discussion of this). The following experiments were designed to
investigate GnRH dose - LH response relationships in acyclic mares
and in cyclic mares during oestrus and dioestrus. Performing dose-
response experiments in cyclic mares posed problems, since both pre-
injection LH levels and LH release induced by a constant GnRH dose
had been observed to vary with day of cycle (See for example
Experiment 11). To compensate for day to day variations in pre-
injection LH levels in each mare, response to GnRH was expressed
not only as area units released but as fractional change from
pre-injection levels (i.e. LH response).

Pre-injection levels
Plotting this expression against GnRH dose given would accurately
describe the GnRH dose-LH response relationship when variations in
pre-injection levels had been caused by changing pituitary
responsiveness (See Figure 38), a situation that would most likely
arise in dioestrus when pre-injection LH levels and pituitary
responsiveness were observed to be highly correlated (See Experi-
ment 11).
EXPERIMENTAL PROTOCOL

A. Experiment 12

Aim; To Study the GnRH Dose-LH Response Relationship in Seasonally Acyclic Mares

Each of 4 mares (Baruch, Oriental Scott, Mrs Oram and True Return) was given 0.5, and 1.0 mg GnRH i.v. and bled at -0.5, 0, 0.5, 1.0, 1.5 and 3 h from injection. The doses were separated by a 3 day interval. Order of doses was not randomised; 1.0 mg being given first to all mares. Serum LH levels were measured by in vitro bio-assay.

B. Experiment 13

Aim; To Study in Dioestrous Mares:

1) The GnRH Dose-LH Response Relationship and


a) Each of 4 Mares (Zany, Lilalee, Safe Dream and Milford Nurse) was given 0.125, 0.25, 0.5, and 1.0 mg GnRH i.v. on days 6, 7, 8, and 9 of dioestrus. On each day, a second injection of the same GnRH dose was given 2 h after the first injection. Order of the 4 doses was randomised with the provision that on each day of the experiment, each mare received a different GnRH dose. Jugular vein cannulation was performed as described for Experiment 7, Section 1, and blood samples were collected at 15 minute intervals for 2 h before and after the first GnRH injection, and for 2 h after
the second injection. Serum LH levels were measured by \textit{in vitro} bioassay.

b) Each of 3 mares (Coming In, Fourth Hall and True Return) was given 0.05, 0.1, 0.2 and 0.4 mg GnRH i.v. on days 2 and 3 (Fourth Hall, True Return) or 4 and 5 (Coming In) of dioestrus. Two doses were given each day at 5-7 h intervals, order of doses was randomised in one mare (Coming In), but not in the other two mares to which doses were given in order of increasing size in an attempt to minimise any "carry-over" effect of a large dose given early in the experiment. Blood samples were collected at -1.0, -0.5, 0, 0.5, 1.0, 1.5 and 2.0 h from each injection. Serum LH levels were measured by radio-immunoassay.

C. Experiment 14

\textbf{Aim: To Study the GnRH Dose-LH Response Relationship in Oestrous Mares}

a) Six cyclic mares (Baylight, Holy Name, Scottish Chat, Comeaway, Annin and Oriental Scott) were given 250 $\mu$g prostaglandin and 10 mg oestradiol 17$\beta$ or oestradiol benzoate in oil i.m once daily for 7-8 days beginning on the day of ovulation. Once daily injections of oestradiol only were continued for a further 6-7 days. Ovarian activity was assessed by palpation \textit{per rectum} at frequent intervals during the experiment.

This treatment was devised by Dr. R.G. Loy\textsuperscript{1} (University of Kentucky, Lexington, Kentucky) and was intended to induce in these mares, stable, high LH levels, and to suppress ovarian activity. Thus, the GnRH dose-LH response relationship could be tested under conditions of high LH secretion rate (as during Oestrus) uncomplicated by changing levels of ovarian steroids which may alter both pituitary responsiveness to GnRH stimulation and patterns of

\textsuperscript{1} Who also did the field work for this experiment.
GnRH secretion.

Each of the mares was given 0.25, 0.5, 1.0 and 2.0 mg GnRH i.v. on the last 4 days of oestradiol treatment. Jugular vein cannulation was performed as described in Section 1, and blood samples were collected at 15 minute intervals for 2 hours before and after GnRH injections. Serum LH levels were measured by radio-immunoassay.

b) Each of 4 dioestrous mares (Safe Dream, Lady Sherelle, Annin and Fourth Hall) was given 250μg of prostaglandin i.m. (Annin was at day 5 of dioestrus, while the other mares were at day 15 of dioestrus). Four days later, mares were teased to determine sexual receptivity and follicular activity assessed by ovarian palpation per rectum. Mares were then given 0.3 mg GnRH i.v. The following day each mare received 0.1 and 0.9 mg GnRH at 5 hour intervals; dose order was randomised. Jugular vein cannulation was performed as described in Section 1 and blood samples collected at -1.0, -0.5, 0, 0.5, 1.0, 1.5 and 2 hours from each GnRH injection. Serum LH levels were measured by radio-immunoassay.

c) Each of 4 oestrous mares (Coming In, Stoat, Fourth Hall and True Return) was given 0.05 and 0.1 mg GnRH i.v. on the second to fourth day of oestrus. Both doses were given on the same day at 5-7 hour intervals; 0.05mg being given first to all mares except Coming In, who received 0.1 mg first. The experiment was repeated 2 days later in all mares except Coming In. All mares were still in oestrus at the second experiment. Ovarian activity was assessed by palpation per rectum on the day of the first (Coming In) or second experiment. Blood samples were collected at -1.0, -0.5, 0, 0.5, 1.0, 1.5 and 2.0 hours from each GnRH injection. Serum LH levels were measured by radioimmunoassay and by in vitro bioassay.
ASSAYS

Because results of Experiment 9 indicated that comparisons of pituitary responsiveness to GnRH stimulation at different cycle stages were not affected by method of assay, bio- and immuno-assay were used interchangeably to measure serum LH in these experiments. However, as a precautionary measure, neither assay method was exclusively used to assess the GnRH dose-LH response relationship in dioestrous or oestrous mares (See Protocol).

CALCULATION OF LH RESPONSE TO GnRH INJECTION

The area of the LH response to single or first GnRH injections was calculated as described earlier in this section. When two injections were given on the same day, response to the second injection was calculated using the following formula:

\[
\text{RESPONSE } 2 = (\text{area under graph of serum LH levels after the 2nd GnRH injection}) - (\text{area under the graph of the extrapolated disappearance curve of the LH response to the first GnRH injection})
\]

This modified formula was used to compensate for the decreasing presence during the second induced response of LH released in the first induced response, and sought to avoid underestimation of the size of the second response. The plasma half-life of equine LH was estimated to be 5 hours, based on results of an earlier experiment. (Irvine, 1979). Occasionally, during oestrus, the response to the first injection plateaued and did not decay as expected before the second injection. In these cases, it was assumed that the first response plateau would be maintained during the second response.
STATISTICAL ANALYSES

Analysis of all experiments was similar. Basically the
effect of GnRH dose on LH response was assessed by analysis of
variance, in which individual mares were considered to be treatment
replicates. Experiment 13a was analysed as a 4x4 Latin Square, in
which effects of dose and order of dose were tested. The effect
of dose order on LH response was also analysed in Experiment 13b,
however, here the Latin Square model was not used since numbers of
mares, doses and orders were not equal. When necessary, treatment
means were compared by Duncan's new multiple range test.

To compensate for day-to-day variation in pre-injection LH
levels in each mare, response to GnRH was also expressed as
fractional change from pre-injection levels. When two injections
were given on the same day, the fractional change to the second
injection was calculated using initial pre-injection levels.
These data were then submitted to analysis of variance as described
above. For each experiment fractional change in LH levels was
plotted against GnRH doses on a linear-linear scale. Fit of the
data to a straight line was then determined by regression
analyses and calculation of the correlation coefficient, r.
EXPERIMENT 12 ACYCLIC MARES

Mean bioactive LH levels (± S.E.M.) in 4 acyclic mares before and at frequent intervals after injection of 0.5 and 1.0 mg GnRH are shown in Figure 39. Pre-injection LH levels, response to GnRH and fractional change in LH levels in each mare are displayed in Table 24.

<table>
<thead>
<tr>
<th>Mare</th>
<th>GnRH dose (mg)</th>
<th>Pre-injection LH (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oriental</td>
<td>0.5</td>
<td>0.49</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Scott</td>
<td>1.0</td>
<td>0.49</td>
<td>3.5</td>
<td>7.1</td>
</tr>
<tr>
<td>Mrs.</td>
<td>0.5</td>
<td>1.2</td>
<td>10.8</td>
<td>9.0</td>
</tr>
<tr>
<td>Oram</td>
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<td>1.9</td>
<td>25.8</td>
<td>13.6</td>
</tr>
<tr>
<td>Baruch</td>
<td>0.5</td>
<td>3.8</td>
<td>10.4</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.5</td>
<td>15.3</td>
<td>3.4</td>
</tr>
<tr>
<td>True</td>
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<td>5.5</td>
<td>4.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Return</td>
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<td>11.1</td>
<td>32.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Mean</td>
<td>0.5</td>
<td>2.7±1.2</td>
<td>6.9±2.2</td>
<td>4.1±1.7</td>
</tr>
<tr>
<td>± S.E.M.</td>
<td>1.0</td>
<td>4.5±2.3</td>
<td>19.3±6.4</td>
<td>6.8±2.4</td>
</tr>
</tbody>
</table>

Mean response (± S.E.M.) induced by 0.5 and 1.0 mg GnRH were 6.9±2.2 units and 19.3±6.4 units respectively, however these could not be shown to be significantly different using analysis of variance (F 4,3 = 4.24, NS). Expressing response as
Figure 39. Mean bioactive LH levels in 4 seasonally acyclic mares before and after injections (at arrow) of 0.5 and 1.0 mg GnRH. Vertical bars denote the standard error of the mean.
fractional change from pre-injection LH levels resulted in treatment means (±S.E.M.) of 4.1±1.7 (induced by 0.5mg GnRH) and 6.8±2.4 (1.0mg GnRH) which were significantly different (\(F_3^1=10.43, p<0.05\)). When plotted as LH response versus GnRH dose on a linear scale, these data fitted well to a straight line passing through the origin (i.e. GnRH dose=0, fractional change in LH levels=0, regression equation: \(y = 6.8x + 0.23, r = 0.993\) (see Figure 55)).

EXPERIMENT 13 DIOESTROUS MARES

A. Serum bioactive LH levels before and after the first injection of 0.125, 0.25, 0.5 and 1.0mg GnRH are shown for each dioestrous mare in Figures 40, 41, 42, and 43. Table 25 displays, for each mare, pre-injection LH levels, LH response and fractional change induced by the 4 GnRH doses. In each mare, pre-injection LH levels varied during the course of the experiment; group means being: day 1 of the experiment = 17.4 units, day 2=28.1 units, day 3=12.0 units and day 4=8.1 units. Analysis of variance showed that the effect of day of experiment on pre-injection LH levels was significant \((p<0.05)\), with levels on day 2 being higher than those on days 3 and 4.

Mean response (±SEM) to 0.125 mg GnRH was 17.2±8.1 units; to 0.25mg=34.7±15.1 units; to 0.5mg=19.0±3.2 units and to 1.0mg=52.2±27.4 units. Analysis of variance failed to show a significant effect of GnRH dose on LH response \((F_3^3=2.77, NS)\). However, response was affected by the order that the doses were given \((F_3^3=7.10, p<0.05)\), with the first injection inducing the greatest response \((dose_1=66.9 units; dose_2=24.5 units; dose_3=13.1 units; dose_4=12.6 units; p<0.05, Duncan's Multiple Range Test)\). Mean fractional change in LH levels (±SEM) induced by 0.125mg GnRH was 0.95±0.17, by 0.25mg=1.8±0.95 by 0.5mg=2.3±0.77 and by 1.0mg=5.1±3.2. Neither GnRH nor order given could be shown to affect
Figure 40. Bioactive LH levels in a dioestrous mare before and after injections of 0.125, 0.25, 0.5 and 1.0 mg GnRH. Doses were given on consecutive days in the order shown at the right of each graph.
Figure 41. Bioactive LH levels in a dioestrous mare before and after injections of 0.125, 0.25, 0.5 and 1.0 mg GnRH. Doses were given on consecutive days in the order shown at the right of each graph.
Figure 42. Bioactive LH levels in a dioestrous mare before and after injections of 0.125, 0.25, 0.5 and 1.0 mg GnRH. Doses were given on consecutive days in the order shown at the right of each graph.
Figure 43. Bioactive LH levels in a dioestrous mare before and after injections of 0.125, 0.25, 0.5 and 1.0 mg GnRH.
**TABLE 25:** Pre-injection bio-active LH levels, LH response and fractional change in LH levels induced by 0.125, 0.25, 0.5 and 1.0 mg GnRH in 4 dioestrous mares

<table>
<thead>
<tr>
<th>Mare</th>
<th>GnRH Dose</th>
<th>Order Given</th>
<th>Pre-injection LH (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
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</thead>
<tbody>
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<td>Lilalee</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>1.0</td>
<td>3</td>
<td>8.1</td>
<td>11.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Zany</td>
<td>0.125</td>
<td>4</td>
<td>12.7</td>
<td>8.1</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
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<td>25.8</td>
<td>31.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
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<td>11.8</td>
<td>20.1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
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<td>9.1</td>
<td>131.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Safe Dream</td>
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<td>1.3</td>
</tr>
<tr>
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<td>1</td>
<td>15.7</td>
<td>66.6</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>34.7</td>
<td>15.3</td>
<td>0.44</td>
</tr>
<tr>
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<td>4</td>
<td>7.1</td>
<td>22.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Milford Nurse</td>
<td>0.125</td>
<td>1</td>
<td>36.2</td>
<td>41.6</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
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<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4</td>
<td>3.3</td>
<td>12.9</td>
<td>3.9</td>
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<tr>
<td></td>
<td>1.0</td>
<td>2</td>
<td>38.6</td>
<td>43.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Response expressed as fractional change ($F_r^2$; dose=1.41, NS; order=2.20, NS). However it should be noted that when mean fractional change was plotted against GnRH dose on a linear scale a straight line passing through the origin resulted (regression equation: $y=4.8x+0.22, r=0.989$; See Figure 55).

Data to compare the effect of 2 successive GnRH injections on LH response were available from each mare only for GnRH doses of 0.125 and 0.5 mg. Table 26 shows the first and second responses to these two GnRH doses in each mare.

1. 0.25 mg: LILALEE - 2nd injection intramuscular, SAFE DREAM - endogenously driven LH pulse in last sampling of first response, MILFORD NURSE - not done; 1.0 mg: SAFE DREAM - 2nd injection intramuscular.
TABLE 26: LH response (in area units) of four dioestrous mares to two successive injections of 0.125 or 0.5 mg GnRH given two hours apart

<table>
<thead>
<tr>
<th>Mare</th>
<th>Response</th>
<th>0.125mg</th>
<th>0.5mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lilalee</td>
<td>1</td>
<td>8.7</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.5</td>
<td>15.8</td>
</tr>
<tr>
<td>Milford</td>
<td>1</td>
<td>41.6</td>
<td>12.9</td>
</tr>
<tr>
<td>Nurse</td>
<td>2</td>
<td>10.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Zany</td>
<td>1</td>
<td>8.1</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Safe</td>
<td>1</td>
<td>10.4</td>
<td>15.3</td>
</tr>
<tr>
<td>Dream</td>
<td>2</td>
<td>9.7</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Mean responses (± SEM) to the successive injections were:
0.125mg: 1. 17.2±8.1 units, 2. 8.8±1.5 units
0.5 mg: 1. 18.9±18.9 units, 2. 12.3±3.8 units

Because of the similarity between doses in mean first and second induced LH responses, data were pooled and responses compared by paired t-test. Although the second response was apparently smaller than the first, this difference was not significant (t=1.86, df=7).

Figure 44 shows serum immunoactive LH in 3 dioestrous mares before and after injection of 0.05, 0.1, 0.2 and 0.4mg GnRH. Pre-injection LH levels, LH responses and fractional change in LH levels induced by the 4 GnRH doses are shown for each mare in Table 27.

Mean response (± SEM) to 0.05mg GnRH was 15.5±2.6 units, to 0.1 mg=22.1±11.3 units, to 0.2mg=12.6±3.5 units and 0.4=9.2±2.3 units. As with the previous group of dioestrous mares, response was not affected by GnRH dose ($F^2_3=7.35, NS$) but was affected by the order that doses were given ($F^2_3=13.05, p<0.05$). Using Duncan's new
Figure 44. Immunoactive LH levels in 3 dioestrous mares before and after injections of 0.05, 0.1, 0.2 and 0.4 mg GnRH.

Each mare received 2 GnRH doses/day on consecutive days

\[ a = 0.05 \text{ mg GnRH} \]
\[ b = 0.1 \text{ mg} \]
\[ c = 0.2 \text{ mg} \]
\[ d = 0.4 \text{ mg} \]

--- = experiment day 1
----- = experiment day 2

HOURS FROM FIRST GnRH INJECTION

---**---
Table 27: Pre-injection immuno-active LH levels, LH response and fractional change in LH levels induced by 0.05, 0.1, 0.2 and 0.4mg GnRH in 3 dioestrous mares

<table>
<thead>
<tr>
<th>Mare</th>
<th>GnRH Dose</th>
<th>Order Given</th>
<th>Pre-injection LH (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>True</td>
<td>0.05</td>
<td>1</td>
<td>5.4</td>
<td>20.7</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2</td>
<td>5.4</td>
<td>12.9</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>3</td>
<td>6.0</td>
<td>12.3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>4</td>
<td>6.0</td>
<td>7.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Return</td>
<td>0.05</td>
<td>3</td>
<td>10.2</td>
<td>13.2</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1</td>
<td>6.7</td>
<td>44.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
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<td>6.7</td>
<td>18.7</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>4</td>
<td>10.2</td>
<td>13.8</td>
<td>1.3</td>
</tr>
<tr>
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<td>4.5</td>
<td>12.5</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1</td>
<td>4.5</td>
<td>8.7</td>
<td>1.9</td>
</tr>
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<td></td>
<td>0.2</td>
<td>3</td>
<td>6.7</td>
<td>6.7</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>4</td>
<td>6.7</td>
<td>6.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Multiple range test, mean first response was found to be significantly greater than the others (mean response: dose 1= 27.2 units, dose 2=6.6, dose 3=10.6 and dose 4=14.6). Mean fractional change in LH levels (± SEM) induced by 0.05mg GnRH was 2.6±0.73; by 0.1mg=3.6±1.5; by 0.2mg=2.0±0.52 and by 0.4mg=1.2±0.09. Both GnRH dose and order given significantly affected this parameter - (F₃, dose=12.22, p<0.05; order=17.25, p<0.025), which was greatest: a) to 0.1mg GnRH, decreasing with increasing dose (see Figure 55), b) to the first injection (dose 1=4.5, dose 2=1.3, dose 3=1.3 and dose 4=2.4, p<0.05, Duncan's multiple range test.)
EXPERIMENT 14 OESTROUS MARES

A. Prostaglandin - Oestradiol Treated Mares

All mares showed behavioural oestrus throughout the experiment and little or no ovarian activity (Holy Name had a small follicle < 15mm in her right ovary throughout the experiment, no significant structures were palpable in the other mares ovaries at any time during oestradiol, or oestradiol and GnRH treatment).

Immunooactive LH levels before and after injection of 0.25, 0.5, 1.0 and 2.0mg GnRH are shown for each mare, in Figures 45 through 50. Table 28 summarises for each mare, pre-injection LH levels, LH response and fractional change induced by the 4 GnRH doses. Pre-injection levels were comparable to maximum oestrous values in this assay system (group mean ± SEM = 70.2±11.8ng/ml). Although not tested statistically, pre-injection levels appeared to be stable during the experiment; no consistent variation with day of experiment being observed. Mean response (±SEM) to 0.25mg GnRH was 28.9±7.9 units; to 0.5mg=22.7±4.1 units; to 1.0mg= 24.5±6.9 units and to 2.0mg=24.9±6.3 units. Mean fractional change in LH levels (±SEM) induced by 0.25mg GnRH was 0.38±0.05; by 0.5mg=0.35±0.06; by 1.0mg=0.34±0.07 and by 2.0mg=0.35±0.05. Analysis of variance failed to show a significant effect of GnRH dose on LH response whether expressed as area units or fractional change.

B. Early Oestrous Mares

Two (Fourth Hall and Safe Dream) of the 4 mares showed behavioural oestrus and marked ovarian activity on the day on which the first GnRH dose was given (i.e. day one of the experiment). Annin came into oestrus on the second day of the experiment, but Lady Sherelle remained unreceptive, with small inactive ovaries, and therefore would seem to have become seasonally acyclic. (See Table 29 for details of ovarian palpations).
Figure 45. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH. (at arrow).
Figure 46. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH (at arrow).
Figure 47. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH (at arrow).
Figure 48. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH.

GnRH dose (mg)

- 0.25
- 0.5
- 1.0
- 2.0

GnRH dose

COMEAWAY

LH (ng/ml)

HOURS FROM GnRH INJECTION
Figure 49. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH (at arrow).
Figure 50. Immunoactive LH levels in a prostaglandin-oestradiol treated mare before and after injections of 0.25, 0.5, 1.0 and 2.0 mg GnRH (at arrow).

GnRH dose
- 0.25
- 0.5
- 1.0
- 2.0

SCOTTISH CHAT

HOURS FROM GnRH INJECTION

NG/mL
Table 28: Pre-injection immuno-active LH levels, LH response and fractional change in LH levels induced by 0.25, 0.5, 1.0 and 2.0 mg GnRH in 6 Prostaglandin - oestradiol treated mares

<table>
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<tr>
<th>Mare</th>
<th>GnRH Dose</th>
<th>Order Given</th>
<th>Pre-injection LH (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
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<td>50.5</td>
<td>16.1</td>
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<td>2.0</td>
<td>4</td>
<td>60.5</td>
<td>21.7</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>29.5</td>
<td>0.32</td>
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</tr>
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<td></td>
<td>0.5</td>
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<td>62.4</td>
<td>22.5</td>
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<td>0.61</td>
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<td>54.9</td>
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<tr>
<td>Chat</td>
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<td>1.0</td>
<td>1</td>
<td>57.3</td>
<td>35.0</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>4</td>
<td>57.8</td>
<td>11.6</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Table 29: Ovarian activity in Experiment 14b mares on Day 1 of the experiment

<table>
<thead>
<tr>
<th>Mare</th>
<th>Ovarian Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth Hall</td>
<td>4.0 cm follicle</td>
</tr>
<tr>
<td>Safe Dream</td>
<td>no follicles distinguishable but ovaries large and active</td>
</tr>
<tr>
<td>Annin</td>
<td>2.5 cm follicle</td>
</tr>
<tr>
<td>Lady Sherelle</td>
<td>ovaries small and inactive</td>
</tr>
</tbody>
</table>

Immunoactive LH levels before and after injections of 0.1, 0.3 and 0.9 mg GnRH are shown, for each mare in Figures 51 and 52. Table 30 summarises, for each mare, pre-injection LH levels LH response and fractional change induced by the 3 GnRH doses.

Mean response (±SEM) to 0.1 mg GnRH was 4.3±1.9 units; to 0.3 mg=6.4±1.9 units and to 0.9 mg=4.6±1.9 units. Mean fractional changes (±SEM) induced by the 3 doses were 0.21±0.05, 0.36±0.11 and 0.23±0.05, respectively. Analysis of variance failed to show a significant effect of GnRH dose on LH response, whether expressed as area units or fractional change. Because of the variability of these mares in amount of ovarian activity, responses of individual mares to the 3 GnRH doses were examined.

In Fourth Hall and Annin, (mares with palpable follicles in their ovaries), increasing GnRH dose did not cause increasing LH response and fractional change in LH levels was a constant. In Safe Dream (mare with large ovaries but no distinguishable follicles), 0.3 mg GnRH caused twice the LH response that 0.1 mg did (fractional change: 0.1 mg=0.21, 0.3 mg=0.41); however, response to 0.9 mg, the second injection of the second day, was smallest of the three responses. Lady Sherelle had inactive ovaries and in her, 0.3 mg GnRH induced 4 times the LH release that 0.1 mg did (fractional change: 0.1=0.17, 0.3=0.61), however, response to 0.9 mg the first dose of the second day, was again poor.
Figure 51. Immunoactive LH levels in 2 oestrous mares before and after injections of 0.1, 0.3 and 0.9 mg GnRH.

FOURTH HALL

Day 1

0.3

Day 2

0.1

0.9

Day 1 = first day of the experiment

ANNIN

Day 1

0.3

Day 2

0.9

0.1

HOURS FROM FIRST GnRH INJECTION

☆ = oestrus

☆ data lost
Figure 52. Immunoactive LH levels in 2 oestrous mares before and after injections of 0.1, 0.3 and 0.9 mg GnRH.

LADY SHERELLE

SAFE DREAM

HOURS FROM FIRST GnRH INJECTION (at arrow)

day 1 = first day of the experiment

= oestrus
Table 30: Pre-injection immuno-active LH levels, LH response and fractional change in LH levels induced by 0.1, 0.3, and 0.9 mg GnRH in 4 "oestrous" mares

<table>
<thead>
<tr>
<th>Mare</th>
<th>GnRH Dose</th>
<th>Order Given</th>
<th>Pre-injection LH (Units)</th>
<th>Response (Units)</th>
<th>Fractional Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth</td>
<td>0.1</td>
<td>2</td>
<td>26.4</td>
<td>9.3</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>1</td>
<td>20.0</td>
<td>6.6</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>3</td>
<td>-</td>
<td>9.8</td>
<td>0.37</td>
</tr>
<tr>
<td>Hall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safe</td>
<td>0.1</td>
<td>2</td>
<td>23.0</td>
<td>4.9</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>1</td>
<td>23.2</td>
<td>9.4</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>3</td>
<td>-</td>
<td>4.7</td>
<td>0.20</td>
</tr>
<tr>
<td>Dream</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annin</td>
<td>0.1</td>
<td>3</td>
<td>-</td>
<td>0.9</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>1</td>
<td>11.0</td>
<td>0.9</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>2</td>
<td>10.1</td>
<td>1.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Lady</td>
<td>0.1</td>
<td>3</td>
<td>-</td>
<td>2.1</td>
<td>0.17</td>
</tr>
<tr>
<td>Shereille</td>
<td>0.3</td>
<td>1</td>
<td>14.3</td>
<td>8.7</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>2</td>
<td>12.4</td>
<td>2.8</td>
<td>0.23</td>
</tr>
</tbody>
</table>

MID TO LATE OESTROUS MARES

Results of ovarian palpations are shown in Table 31

Table 31: Ovarian activity in Experiment 14c mares on the first (Coming In) or second day of the experiment.

FOURTH HALL: Left ovary - 5.0 cm soft follicle

Right ovary - 2.0 cm firm follicle

TRUE RETURN: Ovulated

STOAT: 5.0 cm soft follicle

COMING IN: 4.5 cm softish follicle

Bio- and immuno-active LH levels before and after injection of 0.05 and 0.1 mg GnRH are shown for each mare in Figures 53 and 54. Table 32 compares, in each mare, bio- and immunoassay measurements of pre-injection LH levels, LH response and fractional change induced by the 2 GnRH doses.
Table 32: Bio- and immunoassay measurements of pre-injection LH levels, LH response and fractional change in LH levels induced by 0.05 and 0.1 mg GnRH in 4 oestrous mares.

<table>
<thead>
<tr>
<th>Mare</th>
<th>Day&lt;sup&gt;b&lt;/sup&gt; of oestrus</th>
<th>GnRH dose</th>
<th>Pre-injection LH (units)</th>
<th>Response LH (unit)</th>
<th>Fractional change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bio RIA</td>
<td>RIA</td>
<td>Bio RIA</td>
</tr>
<tr>
<td>True Return</td>
<td>4/7</td>
<td>0.05</td>
<td>25.3</td>
<td>13.3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>7.5</td>
<td>4.7</td>
<td>0.30</td>
</tr>
<tr>
<td>True Return</td>
<td>6/7</td>
<td>0.05</td>
<td>28.2</td>
<td>15.4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>Fourth Hall</td>
<td>4/7</td>
<td>0.05</td>
<td>2.8</td>
<td>3.4</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>4.9</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Fourth Hall</td>
<td>6/7</td>
<td>0.05</td>
<td>6.8</td>
<td>5.0</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>2.4</td>
<td>3.5</td>
<td>0.35</td>
</tr>
<tr>
<td>Coming In</td>
<td>2/5</td>
<td>0.05</td>
<td>20.5</td>
<td>12.3</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>10.4</td>
<td>16.6</td>
<td>0.51</td>
</tr>
<tr>
<td>Stoat</td>
<td>4/8</td>
<td>0.05</td>
<td>3.7</td>
<td></td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>Stoat</td>
<td>6/8</td>
<td>0.05</td>
<td>9.2</td>
<td>4.7</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>b</sup>Expressed as fraction of total number of days in oestrus

Mean immunoactive responses (± SEM) to 0.05 and 0.1 mg GnRH were 4.67 ± 1.12 and 4.67 ± 2.03 units respectively. Mean bioactive responses to the 2 doses were 4.03 ± 1.08 and 4.43 ± 1.61 units. Mean fractional changes induced by the 2 doses were: 0.05 mg = 0.63 ± 0.17 (RIA), 0.65 ± 0.42 (Bio); 0.1 mg = 0.58 ± 0.15 (RIA), 0.50 ± 0.25 (Bio). Analysis of variance could not detect an
Figure 53. Bio- and immuno-active LH levels in 2 mares given 0.05 (a) and 0.1 (b) mg GnRH once or twice during oestrus.

**TRUE RETURN**

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>LH ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
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</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

**COMING IN**

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>LH ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>1</td>
<td></td>
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<tr>
<td>2</td>
<td></td>
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<td>3</td>
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<td>4</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Arrows indicate times of GnRH injection
Day of oestrus is shown as a fraction of the total number of days in oestrus.
Figure 54. Bio- and/or immuno-active LH levels in 2 mares given 0.05 (a) and 0.1 (b) mg GnRH twice during oestrus.

Arrows indicate times of GnRH injection. Day of oestrus is shown as a fraction of the total number of days in oestrus.
effect of GnRH dose or method of assay on LH response, expressed as area units (effect of dose on response: $F^1_6 = 0.08$, N.S., effect of assay on response: $F^1_{10} = 0.65$, N.S.). Since pre-injection levels were the same for both GnRH doses, results of analysis of response expressed as fractional change were identical to those discussed above. The relationship between GnRH dose and LH response varied with mare. In 3 measurements, in each method of assay (see Table 32) doubling GnRH dose approximately doubled response, while in the remaining 3 (Bio) or 4 (RIA) measurements, dose and response were inversely related. However, in most instances the fractional change from pre-injection LH levels induced by GnRH was extremely small and difficult to measure accurately, and presumably accounted for some of the variability in results. Furthermore, it should be noted that although all mares appeared to ovulate normally, pre-injection LH levels were abnormally low by both assay methods in both Stoat and Fourth Hall possibly indicating that these mares were entering seasonal acyclicity (see Ginther, 1979; this experiment was done in May).
DISCUSSION

The results of these dose-response experiments were unexpected and have proved difficult to interpret, posing a formidable tangle of methodological and physiological problems. In acyclic mares, a GnRH dose- LH response relationship was demonstrated in which response was directly proportional to dose, thereby confirming earlier observations (Irvine, 1981). By contrast, in dioestrous mares it was not possible to show statistically a GnRH dose- LH response relationship of any kind due to the significant effect of order of doses on response. However in Experiment 13a in which dose order was completely randomized (thus compensating for the effect of dose order on response), a linear relationship appeared to exist between GnRH dose and MEAN fractional change in pre-injection LH levels. This suggests that failure to demonstrate statistically a GnRH dose- LH response relationship during dioestrus may have been caused by day-to-day variations in response of the cyclic animal, possibly attributable to steroid secretion stimulated by the gonadotrophin surges induced by exogenous GnRH. Alternatively, differences in regimen between anoestrous and dioestrous experiments may have contributed, at least in part. This will be considered in greater detail later. Like dioestrous mares, oestrous mares failed to respond to GnRH in a dose related manner. However, unlike dioestrous mares, the fractional (and absolute) response in LH levels during oestrus was very low (mean fractional response in the 3 experiments = 0.40) with GnRH doses ranging from 0.05 mg to 2.0 mg eliciting similar responses. The dismal results of these experiments with oestrous mares may have been due to inherent difficulties in performing dose-response experiments in a rapidly changing system. For example, alterations in endogenous GnRH secretion between experiments could not be compensated for by expressing response as fractional change (see Figure 38). By the same token, it may not be possible to give oestrous (or dioestrous, see experiment 13) mares 2 GnRH doses/day without significant interaction between doses. Furthermore, the mares used may not have been representative of normal oestrous mares since one group was in "artificial" oestrus induced by prostaglandin and unphysiologically high levels of oestradiol and the other two groups, although in "natural" oestrus, were treated very late in the breeding season. Nevertheless, these mares did respond
to GnRH with the same lowered responsiveness observed in oestrous mares in the breeding season (cf. Experiments 9-11). Thus, although the results of this experiment do not prove that a GnRH dose-LH response relationship does NOT exist in oestrous mares, neither do they provide any indication that oestrous mares can distinguish, in terms of LH response, between GnRH doses ranging from 0.05 to 2.0 mg.

The GnRH dose-LH response relationship has been extensively studied in other species. Results have been summarized in Table 33. In general workers have described the GnRH dose-LH response relationship as being log-linear (i.e. response directly proportional to the log of the dose given; see, for example, Wollesen et al., 1976a & b; Rommler et al., 1979; Gay, Niswender & Midgley, 1970; Cooper, Fawcett & McCann, 1974). However, recalculation of data indicates that in most cases at low GnRH doses (i.e. those inducing <250% increase in LH levels) response is directly proportional to dose (see Table 33). Experiments with cyclic females have had varying success; a GnRH dose-LH response relationship being evident on each day of the rat oestrous cycle (Cooper, Fawcett & McCann, 1974), during the luteal phase in cows (Zolman et al., 1973; Foster, 1978), sheep (Ripple, Moyer, Johnson & Mauer, 1974), and women (Wollesen et al., 1976b), but not during the human follicular phase¹ (Wollesen et al., 1976b) and at no time during the menstrual cycle of the rhesus monkey (Ferin et al., 1974). These results demonstrate once again the problems of working with the dynamic system of the cyclic female. In the experiments with cyclic female rats and sheep, individuals received only one GnRH dose each point on the dose-response.²

¹Rommler et al., (1979) have reported a log-linear GnRH dose-LH response curve in follicular phase women using GnRH doses of 6, 25, 100 and 400 μg. Data were analysed by linear regression of response means. However, inspection of results reveals marked similarity to those of Wollesen et al. (1976a) with responses to 6 and 25 μg virtually superimposable (notice overlap of standard errors of the mean). Had Rommler et al. analysed the experiment using ANOVA as did Wollesen et al., it appears very likely that dose would not be shown significantly to affect response.

²This experimental design, although eliminating error due to interaction between GnRH doses, introduces error due to individual variation in "pituitary responsiveness". To minimise this new error a reasonable number of animals would be required at each dose level, making this approach impractical in the present study due to limited numbers of horses available at the same stage of the oestrous cycle at any given time.
curve being the mean response of different animals. By contrast, when each of 3 luteal phase ewes was given doses of partially purified GnRH\(^1\) ranging from 1 \(\mu\)g to 27 \(\mu\)g, between days 5 and 13 of the oestrous cycle (Day 1 = oestrus), no GnRH dose- LH response relationship could be shown (Reeves, Arimura & Schally, 1970). This observation suggests that in the ewe, as in the mare, LH response may be influenced by repeated GnRH injection. Further support for this idea is provided by the work of Rippel et al. (1974a) in which anoestrous ewes were injected with 100 \(\mu\)g of GnRH at 24, 48, 72, and 96 h intervals. Injections at 96 h intervals caused similar amounts of LH release, however injections at shorter intervals resulted in a progressive decline in induced release. This reduction in response did not seem to be due to depletion of pituitary LH content. On the other hand daily GnRH (100 \(\mu\)g) administration to ovariectomized ewes for 4 days caused only a modest decrease from the initial response (Rippel, 1974a), while administration of small GnRH doses (100 ng) 2, 4 or 7 times daily to rams did not alter LH responses (Lincoln, 1979). The ovaries of anoestrous (and luteal phase) ewes are far from quiescent, natural and administered LH pulses causing sizeable oestradiol release (Baird et al., 1976; McNeilly, O'Connell & Baird, 1982; Scaramuzzi & Baird, 1977). Thus in intact ewes the ovaries may contribute to the decrease in LH response to frequent GnRH injection. Similarly, the marked follicular activity which occurs in luteal phase mares (Ginther, 1979) may have been responsible for the significant effect of order of GnRH dose on LH response observed in Experiment 13, especially since the 2 consecutive GnRH injections given per day caused a steady rise in LH levels which continued to the end of the sampling period. It should be noted that in women in the follicular or luteal phases of the menstrual cycle, LH response was found to be similar to each of 2 GnRH injections of 25 \(\mu\)g given on consecutive days (Rommler et al., 1979), possibly explaining the success of Wollesen et al. (1976b) in showing a GnRH dose- LH response relationship in individuals during the luteal phase but making more puzzling the results of experiments with follicular phase women.

\(^1\)Preparation estimated to be 10% pure, thus amount of GnRH actually administered probably ranged from 0.1 \(\mu\)g to 2.7 \(\mu\)g, well within the linear range of the dose-response curve in rams (Hopkinson et al., 1974)
Because of the inability to demonstrate dose-response curves in oestrous mares in the range of dose given in Experiments 9-11, the "GnRH index" as proposed by Irvine (1980) cannot be used to analyse results. Furthermore, because GnRH doses from 0.05 to 2.0 mg induced similar LH responses, estimates of pituitary responsiveness (i.e. LH release/unit GnRH dose) must be interpreted cautiously in oestrous mares. It appears likely that estimates will vary with dose given. This observation may bring into question other studies in which pituitary responsiveness to exogenous GnRH stimulation has been used to deduce pituitary responsiveness to endogenous GnRH, without establishment of dose-response relationships at each physiological state studied. Prominent amongst such work is the extensive body of research on the human menstrual cycle done by Yen's group at the University of California at San Diego.

In examining the operational characteristics of human gonadotrophs, this group has differentiated between pituitary sensitivity, which is the ability of the gland to immediately release LH in response to an arbitrarily chosen dose of GnRH, and pituitary reserve, which is the ability to respond to prolonged stimulation (Yen et al., 1975). From these somewhat artificial measures (see Rommler, 1978, for criticism of the physiological relevance of Yen's 2-pool model), Yen has made deductions on the control of LH secretion during the human's cycle. It is crucial for the validity of these deductions that the response of the pituitary to exogenous GnRH faithfully predicts the response to endogenous GnRH. However, in follicular phase women no GnRH dose-LH response was found using GnRH doses of 30, 100 or 300 μg (Wollesen et al., 1976b). Furthermore, Yen himself has observed that the ability of the pituitary to distinguish between 10 μg and 150 μg of GnRH (both used by Yen to assess pituitary sensitivity during the cycle; 10 μg: Wang et al., 1976a; 150 μg: Yen et al., 1972) varies with stage of cycle, the 2 doses eliciting similar responses in the early follicular phase and widely differing responses in the late follicular and luteal phases (Wang et al., 1976b). These

1Measured usually as peak change in LH levels induced by a GnRH pulse or as area under the first hour of the LH response curve when GnRH is given as an infusion. "Pituitary responsiveness" measured in the present work as LH response in area units to a small pulse injection of GnRH would probably correspond most closely to Yen's "pituitary sensitivity" (Yen et al., 1975)
observations suggest that Yen's comparative measures of pituitary sensitivity throughout the menstrual cycle may be dose-dependent and may not reflect the response of the gland to endogenous GnRH. Even if dose-response relationships could established for each stage of the cycle, comparisons BETWEEN stages could still be misleading unless the exogenous test dose were carefully chosen to induce LH responses in the linear portion of each dose-response curve (see Figure 56 for worst possible case). This problem can be illustrated by the study of Legan et al. (1980) on the effect of oestradiol treatment on the GnRH dose-LH response relationship in anoestrous ovariectomized ewes. Because dose-response curves in treated and untreated animals had different shapes, these workers observed that the apparent action of oestradiol on pituitary response to GnRH in anoestrus was dependent on the dose of exogenous GnRH given. These comments may also explain the limited success reported by some workers (Vanderkerckove, Dhont & Van Eyck, 1975; Bohm et al., 1978; Mortimer et al. 1976; Jewelewicz et al., 1974) in applying the GnRH stimulation test to diagnose causes of ovarian dysfunction in women.

The ability of oestradiol to blunt, at least initially, LH response to exogenous GnRH has been well documented in several species (see, for example: monkeys, Knobil, 1974; human, Keye & Jaffe, 1974; rat, Libertun, Orias & McCann, 1974: Negro-Vilar, Orias & McCann, 1973; sheep, Goodman & Karsch, 1980). However, in rat pituitary cell culture oestradiol has been reported to have only a stimulatory effect on LH response to GnRH (Labrie et al., 1978; Mukhopadhyay et al., 1979). Most provocatively, a recent report by McNeilly et al. (1982) has shown that when pulses of ovine LH are given intravenously to anoestrous ewes, the area of the resulting plasma pulses declines to be almost undetectable as oestradiol levels rise. This observation could suggest a major and possibly steroidally mediated modification of the peripheral metabolism of LH that has been undetected by previous studies using ^125_{I}-ovine LH (Akbar, Nett & Niswender, 1974). If the report of McNeilly is verified the serum LH response to exogenous GnRH may not be related to pituitary LH release. One further consideration to be made when measuring LH response to exogenous GnRH is that in
species in which the nature of serum LH has been observed to
vary with cycle (e.g. rhesus monkey, human and horse) it might
be prudent to verify immunological assessments of pituitary
responsiveness with bioassay (see, for example, Experiment 9).

Despite the many pitfalls of experiments in which exogenous
GnRH has been used to assess pituitary responsiveness, recent
work in the rhesus monkey has suggested that the conclusions
reached about the changing pattern of responsiveness during the
cycle could be essentially correct. It has been reported
(Knobil, 1980) that in female monkeys deprived of endogenous
GnRH by arcuate nucleus lesion (Plant et al, 1978) ovulatory
menstrual cycles could be restored by administering an unvarying
pattern of exogenous GnRH pulses (lµg/min for 6min once hourly).
Serum LH and FSH levels were observed to approximate the pattern
of the normal cycle. Since GnRH input was constant to these
animals, it seems most likely that changes in pituitary
responsiveness to stimulation - similar to those predicted by
earlier work (Knobil, 1974) - were regulating gonadotrophin levels
(although results may also have been produced by secretion around
the time of ovulation of LH molecules with a longer half-life, or
alterations in peripheral metabolism). On the other hand,
preliminary reports in humans have indicated mixed results in
treating hypothalamic amenorrhea with a constant pulsatile GnRH input;
with increased doses or hCG injection frequently required to
induce ovulation and/or normal corpus luteum function (Zarate
et al, 1973; Nillius, Fries & Wide, 1975; Nillius and Wide, 1975;
Leyendecker, Wildt and Hansmann, 1980; Crowley and McArthur,

In conclusion, then, extreme caution should be used in
interpreting experiments in which the LH response to exogenous
GnRH has been used to predict that to endogenous GnRH. With
regard to the present work in the horse, a GnRH dose-LH response
relationship must be demonstrated in oestrous mares if the
relative importance of changes in pituitary responsiveness and
endogenous GnRH secretion in producing the ovulatory LH surge is
to be assessed. By contrast, in dioestrous mares, a linear-linear
GnRH dose-LH response relationship appears to exist. If this
relationship can be verified in further experiments, it may be
possible to use Irvine's GnRH index at this stage of the cycle,
to evaluate the role of changing GnRH secretion in generating
the mare's mid-cycle FSH surge (Evans and Irvine, 1975).
Figure 55. Summary of Experiments 12-14: GnRH dose-LH response relationship in cyclic and seasonally acyclic mares.

ACYCLIC MARES

\[ r = 0.993 \]

bars indicate standard error of the mean; when not shown, this error was smaller than the width of the symbol marking the data point.

DIOESTROUS MARES

a). \[ r = 0.989 \]

b). \[ r = 0.969 (0-0.1 \text{ mg}) \]

c). \[ r = 0.959 (b), \text{GnRH doses 0-0.3 mg} \]
[ r = 0.823 (c) ]

OESTROUS MARES

a). \[ r = 0.486 \]

b). and c). \[ r = 0.959 (b), \text{GnRH doses 0-0.3 mg} \]
[ r = 0.823 (c) ]
Comparing oestrous and dioestrous responses to exogenous GnRH, it would be concluded that the pituitary is more responsive to stimulation during dioestrus than oestrus (i.e. LH release/unit GnRH at dioestrus > LH release/unit GnRH at oestrus). In fact, pituitary responsiveness to ENDOGENOUS GnRH is greater at oestrus than dioestrus.
Table 33: Relationship between GnRH dose and LH response in several species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Response Metameter</th>
<th>GnRH dose d</th>
<th>LH Response</th>
<th>r*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep (ram)</td>
<td>Average LH increase during 1st 0.5 h post GnRH</td>
<td>0</td>
<td>0</td>
<td>0.996</td>
<td>Hopkinson, Pant and Fitzpatrick (1974)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.125 µg</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>13.6</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.50</td>
<td>24.0</td>
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<tr>
<td></td>
<td></td>
<td>1.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep (luteal phase ewe)</td>
<td>Area under LH response curve</td>
<td>0</td>
<td>0</td>
<td>0.999</td>
<td>Rippel, Moyer, Johnson and Mauer (1974)</td>
</tr>
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<td></td>
<td>5.0 LL</td>
<td>13.6</td>
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<td>Peak LH increase Hypothalamic extract</td>
<td>0</td>
<td>0</td>
<td>0.999</td>
<td>Gay, Niswender and Midgley (1970)</td>
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<tr>
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<td>0.125**</td>
<td>1.25</td>
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<td></td>
<td>2.0 LL</td>
<td>9.48</td>
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<tr>
<td>Sheep (ovariectomised ewe)</td>
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<td>0</td>
<td>0.992</td>
<td>Legan, Goodman, Ryan, Foster and Karsch (1980)</td>
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<td>Response</td>
<td>GnRH dose</td>
<td>LH Response</td>
<td>r*</td>
<td>Reference</td>
</tr>
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<td>----------</td>
<td>-----------</td>
<td>-------------</td>
<td>----</td>
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<tr>
<td>Rat (intact female)</td>
<td>Peak LH increase</td>
<td>hypothalamic extract</td>
<td>am</td>
<td>pm</td>
<td>I</td>
</tr>
<tr>
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<td>0</td>
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<tr>
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<td></td>
<td>50 µl</td>
<td>4.1</td>
<td>7.2</td>
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<tr>
<td></td>
<td></td>
<td>100 µl</td>
<td>7.1</td>
<td>14.1</td>
<td>6.8</td>
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<tr>
<td>Cattle (bull)</td>
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<td></td>
<td>3.0 \text{LH}</td>
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<tr>
<td>Cattle (early luteal phase cow)</td>
<td>Peak LH increase</td>
<td>0</td>
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</tr>
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<td></td>
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<td>5 µg</td>
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<td></td>
<td>20</td>
<td>6.8</td>
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<td></td>
<td></td>
<td>80</td>
<td>10.6</td>
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<tr>
<td>Cattle (luteal phase cow)</td>
<td>Area under LH response curve</td>
<td>0</td>
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</tr>
<tr>
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<td>Response Metamer</td>
<td>GnRH dose[^d]</td>
<td>LH Response</td>
<td>r[^*]</td>
<td>Reference</td>
</tr>
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<tr>
<td>Miniature pig (intact and castrate male)</td>
<td>Area under LH response curve</td>
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<td>0</td>
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<td>Pomerantz, Ellen-dorff, Elsaesser, König and Smidt (1974)</td>
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<tr>
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<td>7.2</td>
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<td>Rebar et al. (1973)</td>
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<td>1 µg</td>
<td>1.05</td>
<td>0.34**</td>
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<td></td>
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<tr>
<td>Human (man)</td>
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<td>0</td>
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<td>0</td>
<td>Wollesen, Swerdloff and Odell (1976a)</td>
</tr>
<tr>
<td></td>
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<td>1.7</td>
<td>0.982</td>
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<td>30</td>
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<td></td>
<td></td>
<td>100 LL</td>
<td>5.1</td>
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<td></td>
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<tr>
<td>Human (luteal phase woman)</td>
<td>Area under LH response curve</td>
<td>0</td>
<td>0</td>
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<td>Wollesen, Swerdloff and Odell (1976b)</td>
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<tr>
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<td>30 µg</td>
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<td>0.870***</td>
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<td></td>
<td>300</td>
<td>7.6</td>
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[^d]: All doses were of synthetic GnRH except where indicated.
[^*]: Correlation coefficient when data were fitted to linear dose-linear response plot.
[^*]: Authors do not consider response to be significantly different to 0.
[^**]: Poor fit to linear linear plot due to large size of GnRH dose given (cf results with men). Results have been included here for completeness.
[^LL]: Larger dose to which induced response would not be expected to be directly proportional. Point has not been included in the linear regression.
[^e]: The point 0.0 has been included in the linear regression because it was assumed that had no GnRH dose been given, there would have been no LH response.
REFERENCES


GINTHER, O.J. (1979) Reproductive Biology of the Mare; Basic and Applied Aspects. Published by the author, Cross Plaines, WI, U.S.A.


NILLIUS, S.J. & WIDE, L. (1975) Gonadotrophin-releasing hormone treatment for induction of follicular maturation and ovul-


