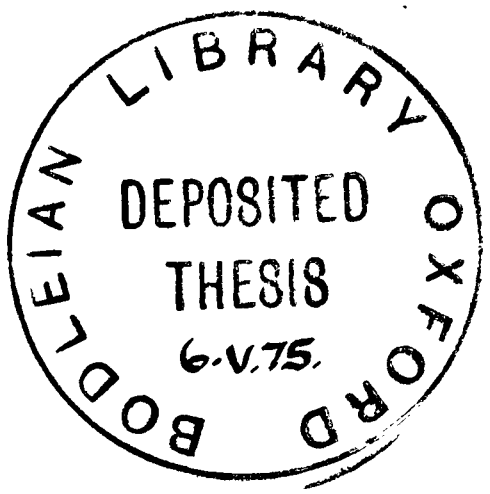
SOME ASPECTS OF THE HYPOTHALAMIC CONTROL OF PITUITARY GONADOTROPHIN SECRETION.

A thesis submitted for the

Degree of Doctor of Philosophy

by

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1974

ABSTRACT

The development of that branch of physiology known as neuroendocrinology has followed the increasing awareness that environmental influences, sensed through the exteroceptors of the central nervous system, play an important part in the control of reproductive and other endocrinologically mediated activity in animals. The implication of pituitary gland activity in these processes in the rat by Smith (1927, 1932), who developed a satisfactory technique for hypophysectomy, led to a search for the connections between the central nervous system and the pituitary gland. It was soon established that the posterior lobe of the pituitary is controlled by neuronal pathways passing from the hypothalamus to that lobe, and investigation of control of the anterior lobe of the gland centred at first around the establishment of a similar neural pathway.

The discovery that the anterior pituitary is almost devoid of nerve fibres, and the failure of investigators to establish any form of autonomic nervous control drew attention to the blood vessels in the pituitary stalk first adequately described by Popa and Fielding (1930, 1933). These vessels offered an alternative control pathway to the anterior lobe and a number of investigators in the 1930's (Hinsey & Markee, 1933; Friedgood, 1936; Hinsey, 1937; Harris, 1937; Haterius, 1937) put forward a tentative hypothesis of humoral control of the anterior pituitary. By 1944, Harris was able to state the neurohumoral hypothesis confidently

in the light of the accumulation of a good deal of supporting evidence.

One of the most profitable techniques used by investigators in experiments on the hypothalamo-hypophysial axis has been electrical stimulation in the central nervous system with concomitant observation of changes in activity of extra-neural structures. Early experiments were crude, Marshall and Verney (1936) applying diffuse electrical stimuli to the heads or lumbar spinal cords of rabbits, and Harris (1936) carrying out similar experiments on rats. In both cases, these experiments indicated that some neural structures in the head had been excited, with consequent ovulation in the rabbits and pseudopregnancy in the rats.

The crudity of the electrical stimulation techniques used at that time meant that close and detailed study of small areas of the central nervous system was impracticable. With the development of electronic stimulators capable of producing accurate, stable trains of stimuli, it became possible to examine hypothalamic areas which might be involved in control of anterior pituitary secretion. A great variety of electrode materials, stimulus waveforms and parameters of stimulation have been used. Frequently, scant regard has been paid to the differentiation of electrical stimulation from electrical lesion production.

Many studies have been performed on the activation of ovulation or of luteinising hormone (LH) secretion by the anterior pituitary in

female rats after electrical stimulation in the diencephalon, and it became evident that medial preoptic area was involved (Critchlow, 1958). There are few reports however, of activation of LH release from the anterior pituitary consequent upon hypothalamic stimulation in male animals. No event comparable to ovulation was observable in male animals, so Moll and Zeilmaker (1966) and Quinn (1966) resorted to transplanting ovaries into castrated males. Direct current (d.c.) stimulation in the medial preoptic area resulted, in both these studies, in changes in the ovarian transplants suggestive of ovulation. With the advent of assays, and in particular radioimmunoassay for LH (Niswender, Midgley, Monroe & Reichert, 1968) it has become possible to study the effects of medial preoptic area stimulation in normal male rats, using a variety of electrode materials and stimulation parameters.

The present studies provide direct evidence for the release of LH from the anterior pituitary gland consequent upon electrical stimulation of the medial preoptic area of the normal male rat, and confirms the preliminary studies of Burger, Fink & Lee (1972) who achieved rises greater than 400% in peripheral plasma LH concentration in three male rats after medial preoptic area stimulation. The optimal parameter for alternating current (a.c.) square wave stimulation are frequency 60 Hz, pulse amplitude 0.5 mA and pulse duration 0.5 - 1.0 msec. Release of LH occurs when both steel and platinum twin unipolar electrodes are used, although the effects of a.c. stimulation with steel electrodes, which results in deposition of ferric ions in the tissues, are prolonged

in comparison to the effects of a.c. stimulation with platinum electrodes.

Direct current (d.c.) stimulation of the medial preoptic area with steel electrodes is highly effective in causing increased secretion of LH by the anterior pituitary. Again, there is considerable deposition of ferric ions from the electrode tips. D.c. stimulation with platinum electrodes is ineffective however, which provides further evidence that deposition of metallic ions in nervous tissue has potent stimulatory effects.

The results of the variety of stimulus procedures used in the present study suggest that as in the female rat, the medial preoptic area is involved in the control of gonadotrophin release in the male. While there is no cyclical waxing and waning of gonadotrophin release in the male rat, there is evidence that normal male sexual behaviour is dependent on an intact preoptic area (Lisk, 1968; Giantonio, Lund & Gerall, 1970).

In considering the results of the present and previous stimulation experiments, caution is urged such that when reporting stimulation and lesion experiments not only should electrical parameters including coulombage of pulses and pulse trains be reported, but also the physical and chemical characteristics of the electrodes themselves should be described.

Another form of evidence in support of the neurohumoral hypothesis of control of the anterior pituitary gland has been the discovery in extracts of hypothalamic tissue of substances capable of affecting the secretion of anterior pituitary hormones. Evidence for the occurrence of hypothalamic substance(s) which act so as to release gonadotrophins was produced by Harris and co-workers (Harris, 1960; Campbell, Feuer, Garcia & Harris, 1961) who showed that infusion of extracts of the hypothalamic median eminence directly into the pituitary gland was effective in inducing ovulation in rabbits. Characterisation of these so-called hypophysiotrophic factors depended on the development of reliable, specific, sensitive, direct assay methods.

After Schally and his co-workers (Schally, Arimura, Bowers, Sawano & Redding, 1968) had purified hundreds of thousands of porcine hypothalami, they were able to extract and isolate a substance capable of releasing LH and FSH from the anterior pituitary. Structural determination of this material as a decapeptide followed, and it was synthesised (Matsuo, Arimura, Nair & Schally, 1971). Similar work with ovine hypothalami was carried out by Guillemin and co-workers (Amoss, Burgus, Blackwell, Vale, Fellows & Guillemin, 1971). The availability of synthetic luteinising hormone-releasing factor (LH-RF) as it was called meant that assays using synthetic LH-RF as a standard became available.

In the present studies, in-vitro bioassays using short-term

incubation of anterior pituitary glands from normal male animals (3-4 pituitaries/flask) and from ovariectomised animals (1 pituitary/flask) pretreated with oestrogen and progesterone (Ramirez & McCann, 1963) have been established. In both cases, addition of doses of synthetic LH-RF ranging from 1.9 ng/ml incubation medium to 33.0 ng/ml medium caused release of significant amounts of both LH and FSH over a six hour incubation period. Satisfactory dose-response curves were established in both assays.

The principal desirable characteristics of an assay method are specificity, precision, sensitivity, repeatability, convenience and economy. In both assay systems LH and FSH release is caused by high concentrations of potassium, but the concentrations of potassium in natural materials tested in the assay are sufficiently low so as to not interfere in this respect. In the female assay system monoamines and octapeptides at dose levels that might be expected to occur in extracts of natural materials do not cause release of LH or FSH.

A number of synthetic fragments of LH-RF have been tested for LH and FSH releasing activity in the female assay system. Only the nonapeptide (LH-RF 1-9) exhibited consistent activity. The importance of the entire chain length of LH-RF for full gonadotrophin releasing activity is underlined by the fact that removal of the C-terminal amino acid results in depression of releasing activity by up to 95%.

Precision and sensitivity of both assay systems are satisfactory, but there is considerable between assay variation. In terms of economy it is evident that the use of pituitaries from oestrogen-progesterone treated ovariectomised animals enables considerable saving in numbers of assay animals required.

A radioimmunoassay (RIA) first described by Nett, Akbar, Niswender, Hedlund & White (1973) has been established in this laboratory using ^{125}I-labelled LH-RF as tracer. It appears to be specific for the decapeptide LH-RF, and it is clear that it is appropriate to measure LH-RF concentrations in a variety of biological materials using this RIA. Regrettably, clear data on between-assay variability is lacking in the assays described, though the quality control parameters observed in the assay suggest that it is reasonably "in control". The fact that intact plasma contains constituents which affect binding of hormone to antiserum means that care must be taken when interpreting results, especially when the LH-RF concentration is low.

Measurements have been made of LH-RF concentration in plasma of post-menopausal women and no elevation has been observed despite the fact that LH secretion is generally high after the menopause (Yen, Tasi, Naftolin, Vandenberg & Ajabor, 1972). It is apparent also that coital activity in the male rat has no discernable effect on peripheral plasma LH-RF concentration in the first 20 min. after coitus.

The availability of in-vitro bioassay and radioimmunoassay for LH-RF has prompted an examination of hypophysial portal blood collected from the cut pituitary stalk of rats under various experimental conditions. The work of Fink and co-workers (Fink, Nallar & Worthington, 1966, 1967; Fink 1967a, 1967b) showed that LH-RF activity was present in hypophysial portal plasma using in-vivo assays while Porter and his co-workers (Kamberi, Mical & Porter, 1969, 1970; Ben-Jonathan, Mical & Porter, 1973) have used in-vitro assays to detect LH-RF activity in hypophysial portal blood.

In the present studies LH-RF has been detected in ultra-filtrates of pituitary stalk plasma of male animals by in-vitro assay. While it was only possible to achieve parallelism of dose-response curves between synthetic LH-RF and stalk plasma ultrafiltrate in one of six assays, it is clear that stalk plasma ultrafiltrate contains material capable of releasing both LH and FSH from pituitary glands in-vitro.

By RIA, LH-RF was detected in pituitary stalk plasma of both male and female rats. With respect to the male, the evidence that stimulation of the medial preoptic area leads to significant increases in LH-RF secretion complements and accounts for the rises in peripheral plasma LH consequent upon medial preoptic area stimulation.

In the female, while the contents of LH-RF in stalk plasma collected at pro-oestrus and metoestrus are somewhat higher than those in stalk plasma collected at oestrus and dioestrus, the differences are

not statistically significant. The surge of LH secretion on the afternoon of pro-oestrus and the smaller rise in peripheral LH concentration at metoestrus in females, may therefore be due to changes in sensitivity of the pituitary gland to constant or only slightly increased levels of LH-RF in hypophysial portal blood at those times.

As in the male, electrical stimulation markedly increased the contents of LH-RF in pituitary stalk blood in the female. There is no significant evidence that the sensitivity of the hypothalamo-median eminence-portal vessel axis varies during the oestrous cycle, though Fink & Aiyer (1974) find that the sensitivity of the entire hypothalamo-hypophysial axis to electrical stimulation is markedly enhanced on the afternoon of pro-oestrus.

In conflict with the results of others (Burger, Fink & Lee, 1972; Ben-Jonathan, Mical & Porter, 1973) no evidence of increased secretion of LH-RF was found in castrated female rats when compared with intact dioestrous controls.

The electrodes and the optimal a.c. stimulation parameters used in the present studies do not appear to have deleterious affects, as female rats continue to cycle with electrodes implanted in the preoptic area, and LH-RF secretion increases markedly in both of two 30 min. stimulation periods separated by a rest period of 30 min.

While preoptic area stimulation can more than double pituitary stalk blood flow rates, such blood flow increases cannot account for the magnitude of increases in LH-RF secretion during stimulation.

The major problem encountered in these studies has been the enormous disparity between estimates of LH-RF concentration in pituitary stalk plasma made by bioassay and RIA methods.

Explanations for this disparity include the possibilities that the results either of the bioassay or of the RIA are artefactual, or that the synthetic preparation of LH-RF is not identical to the "physiological" endogenous LH-RF, or that endogenous LH-RF exists in portal blood either in "pro-hormone" form or bound to a plasma protein.

ACKNOWLEDGEMENTS

I owe a debt of gratitude to the late G.W. Harris C.B.E., F.R.S., first for the hospitality which he offered me in 1971, and secondly for his guidance and criticism prior to his death in 1971. I should like to thank Professor A.G.M. Weddell for his interest and advice and for his kind continuance of the hospitality of the Department of Human Anatomy over the past three years.

The experiments described herein have been carried out under the scientific supervision of Dr. G. Fink to whom I am vastly indebted for his inspiration and advice. Dr. K. Brown-Grant and Dr. H. Charlton freely gave advice on some experiments, and kindly loaned facilities and equipment for some experiments. Dr. S. Bradbury helped with advice on image-analysing computing and histological techniques, and Dr. T.P.S. Powell gave advice on histological techniques.

This work would not have been possible without the assistance of Mrs. S.A. Windebank and in particular that of Miss V. Langford who materially assisted in every one of the experiments described.

This work was carried out under the tenure of a Rhodes Scholarship, and technical expenses were defrayed by a grant to the author from the Medical Research Council, No. G 971/378/C. Standard

hormone preparations were the gift of Imperial Chemical Industries Ltd., the National Institutes of Health and the National Institute for Arthritic and Metabolic Disease of the U.S.A. and Dr. G.D. Niswender.

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CHAPTER 1

INTRODUCTION

1.1. The central nervous system and the anterior pituitary gland

Indications that the nervous system exercises over-riding control over reproductive function were available long before meaningful experimentation on brain-endocrine relationships was possible. In 1675, Martin Lister wrote in a letter to John Ray that swallows will lay more eggs than is usual if one egg is removed daily from the nest during the laying period. Haighton, in 1797, observed in a paper entitled "An experimental inquiry concerning animal impregnation" that ovulation followed coitus in the rabbit. Heape, in 1905, timed the interval between mating and ovulation in the rabbit, giving a figure of about 10 h., and suggested that a "generative ferment" in the general circulation was responsible for this "reflex".

In the early part of this century, it became evident that light, and in particular, day-light, had readily observable effects on reproductive function. Rowan (1926) showed that the testes of finches subjected to increased artificial illumination during the winter months were larger than those in finches exposed only to natural light.

Other environmental factors were implicated in the control of reproductive activity and Marshall summarised much information in two important reviews (Marshall, 1936, 1942), pointing out that conditions of nutrient intake, ambient temperature, the presence of eggs or young,

the presence of a mate (even without physical contact) and psychological changes may also affect the sexual and reproductive activity of species from the invertebrates to vertebrates and man. Marshall concluded that this observational data suggested that environmental stimuli act through the exteroceptors and reflex pathways in the central nervous system to effect secretion by the anterior pituitary gland. It should be pointed out that centuries of domestication of a variety of animals had led to many empirical observations on reproductive activity made by husbandmen concerning the influences of environment on the breeding of their charges.

The fact that such effects of exteroceptive stimuli on reproductive activity might be mediated through the anterior pituitary had been demonstrated by Smith (1927, 1932) who developed a satisfactory technique for hypophysectomy in the rat which avoided damage to the hypothalamus. He showed that removal of the pituitary gland retarded growth, and caused atrophy of the ovaries or testes, thyroid gland, and adrenal cortices.

The anatomical arrangement of the hypothalamus and the pituitary suggested that the controlling influence might traverse the pituitary stalk and investigators carried out experiments designed to identify precisely the way in which the brain exerted control over the anterior pituitary. A direct nerve supply via the stalk was the most plausible and obvious suggestion, and the possibility

of sympathetic innervation via the blood vessels or parasympathetic supply via the petrosal nerves was also mooted. Dandy (1913) in investigations of the sympathetic supply noted that few sympathetic fibres passed to the pituitary, and that large areas of the anterior pituitary were free of nerve fibres. Cannon, Newton, Bright, Menkin & Moore (1929) showed that sympathectomised cats could reproduce normally, and Brooks (1938) confirmed this in rabbits. Hinsey and Markee (1933), and Haterius (1934) showed that reflex ovulation could occur after section of the cervical sympathetics. Hair and Mezen (1939) demonstrated that the petrosal nerves are of little importance for anterior pituitary function when they found that reflex release of gonadotrophic hormone following coitus in the rabbit was unaffected by destruction of the petrosal nerves.

A nerve supply from the hypothalamus was also investigated. Cajal (1911) described nerve terminations in the pituitary stalk, in the neural lobe of the pituitary and in the intermediate lobe (curiously he believed that the neural lobe was a sensory organ). Although some workers described the passage of numerous fibres from the hypothalamo-hypophysial tract to the anterior pituitary, it is likely that reticular connective tissue fibres had been mistaken for nerve fibres. In a survey of the pituitary glands of many vertebrate types Green (1951) concluded that "in none of the animals studied has an innervation of the pars distalis been found." However, in 1952,

Vasquez-Lopez & Williams described what they believed were nerve bundles running along the pituitary stalk in the direction of the anterior pituitary. They commented that "the innervation of the rat adeno-hypophysis cannot be regarded as proved". In fact, such an innervation was to be decisively disproved with the advent of electron-microscopy.

Attention in the 1930's was focussed on the hypophysial portal vessels which were known to exist before their significance was realised. A number of workers (Hinsey & Markee, 1933; Friedgood, 1936; Hinsey, 1937; Harris, 1937; Haterius, 1937; Brooks, 1940, Taubenhaus & Soskin, 1941) tentatively put forward the view that a humoral transmission of stimuli from the hypothalamus to the anterior pituitary was possible. In 1944, Harris stated the neurohumoral hypothesis with more confidence:

" . . . that the supraoptic nuclei and probably other nuclei not yet accurately located, innervate the neurohypophysis (median eminence, infundibular stem and infundibular process) and that this functional unit in turn activates the lobus glandularis by chemical transmission through the hypophysial portal vessels . . . ". In 1947, Green and Harris restated the hypothesis clearly. Evidence supporting this explanation of anterior pituitary control by the central nervous system soon accumulated, the main lines of evidence being:

(A) Anatomical, in that

(i) it was found by direct microscopic investigation in living animals that the blood in the hypophysial

portal vessels flowed from the hypothalamus along the stalk into the pituitary gland, and that

(ii) hypophysial portal vessels were found to be a constant feature of the vertebrate pituitary gland, and that

(iii) even in forms in which thick connective tissue septum separated the two lobes of the pituitary gland (e.g. the Cetacea; Harris, 1950a), these vessels still connect the hypothalamus to the anterior pituitary by passing round the anterior end of the septum.

(B) Physiological, in that

(i) electrical stimulation of certain parts of the nervous system (but not direct stimulation of the pituitary gland itself) causes release of adenohypophysial hormones, the hypophysial portal vessels in the pituitary stalk being essential for this mechanism to occur, and that

(ii) transplants of the anterior pituitary function properly only when in contact with the median eminence, and that

(iii) substances have been extracted from the median eminence of the hypothalamus which are capable of affecting the secretion of adenohypophysial hormones, and that

(iv) substances have been extracted from hypophysial portal blood which are capable of affecting the secretion of adenohypophysial hormones.

Fuller accounts of much of this evidence appear in the initial

sections of Chapters 2, 3 and 4.

The aims of the studies reported herein were to investigate the hypothalamic control of pituitary gonadotrophin secretion, initially by an examination of the discharge of gonadotrophins into the general circulation consequent upon electrical stimulation of the diencephalon (Chapter 2). In addition, the recent characterisation and synthesis of the luteinising hormone-releasing factor (LH-RF) has facilitated the development of quantitative bioassay and radio-immunoassay of LH-RF (Chapter 3). Finally, hypophysial portal blood has been examined for the presence of LH-RF under a variety of experimental conditions (Chapter 4). A summary of the conclusions of these studies appears in Chapter 5.

Note on nomenclature. A variety of names and abbreviations have been coined for the luteinising hormone-releasing factor (LH-RF). Others include gonadotrophin-releasing hormone (Gn-RH), gonadotrophin-releasing factor (Gn-RF) and luteinising hormone-releasing hormone (LH-RH). In the present studies the term luteinising hormone-releasing factor is preferred, and the abbreviation used is LH-RF, though in some Figures the abbreviation LRF appears.

CHAPTER 2

PARAMETERS OF ELECTRICAL STIMULATION

IN THE DIENCEPHALON

2.1. SUMMARY

After consideration of the use of electrical stimulation of the central nervous system in the study of reproductive physiology, experiments are described in which the most appropriate parameters for stimulation of areas of the diencephalon were investigated. The response to such stimulation measured was the change in peripheral plasma gonadotrophin concentration at various times during and after the application of such stimuli. The results of these experiments are discussed against a background of previous experimentation, and recommendations are made regarding the use of electrical stimulation techniques as an investigative tool.

2.2. INTRODUCTION

The use of electrical stimulation of the brain with concomitant recording of various changes (or their absence) in neural, somatic, or behavioural activity has been a powerful tool in the investigation of the structural and functional organisation of the nervous system. All the same, gross excitation of cell and fibre masses is still a crude way of asking questions of the nervous system, and it must be expected that the answers will be only approximations. The paragraphs that follow present findings obtained in an investigation of the various parameters to be taken into account in the stimulation of some neural structures concerned with reproductive activity.

2.3. HISTORY

Stimulation of the hypothalamus with observation of any resultant change in the macroscopic or microscopic appearance of peripheral target organs such as the ovary or testis is a somewhat tedious procedure. The relatively slow rate of oestrous cycles, the slow formation and maturation of follicles and corpora lutea, and the slow rate of spermatozoal production and maturation make it seem unlikely that short-lived stimulation of the hypothalamus would produce readily observable peripheral changes. One of the fastest ovarian reactions is that of ovulation in the rabbit which occurs 9 - 11 hours post-coitum. Marshall and Verney (1936) employed this relatively quick reaction when they applied diffuse electrical stimuli to the heads or lumbar spinal cords of female rabbits. Ovulation and pseudopregnancy resulted in a large proportion of animals. Harris (1936) extended this work to rats, inducing active corpora lutea and pseudo-pregnancy, again by applying diffuse electrical stimuli to the head. While the techniques of stimulation used were crude, these results indicated that some neural structures had been excited, and that this in turn had by some means stimulated the anterior pituitary gland to produce gonadotrophins.

Harris (1937) then proceeded to localise the neural structures involved by applying electrical stimuli directly to the hypothalamus and pituitary gland in anaesthetised rabbits. The relative crudity

of his stimulation technique at this stage was underlined by his findings that not only did stimulation of the tuber cinereum and posterior hypothalamus result in ovulation, but also stimulation of the anterior pituitary gland itself. Harris suggested in the light of later work that the ovulatory responses consequent upon direct stimulation of the pituitary gland were due to the ether anaesthesia. It is rather more likely that it was due to spread of his stimulus from the anterior pituitary to the tuber cinereum. This viewpoint is supported by Markee, Sawyer and Hollinshead (1946) who stimulated the pituitary gland by a pharyngeal or a temporal approach, and the hypothalamus through the superior orbital fissure in ether anaesthetised rabbits. They found that electrical stimulation of the pituitary did not result in ovulation unless there were "signs of spread of the stimulus", whereas stimulation of the hypothalamus at a lower voltage than that used for direct pituitary stimulation resulted in ovulation in three of four animals. Harris (1948), using remote control stimulation of the tuber cinereum in unanaesthetised rabbits found that three minute periods of stimulation might be followed by a full ovulatory response, whereas similar stimuli applied to the anterior pituitary for up to $7\frac{1}{2}$ hours were not followed by any ovarian response.

By mid-century then, electrical stimulation techniques had been employed to provide one form of evidence that the hypothalamus

was implicated in the control of the anterior pituitary gland. From this and other evidence Harris (1955) comments:

"Returning to the effect of electrical stimulation in causing gonadotrophin secretion and ovulation, the position seems to be that stimulation of the tuber cinereum may produce this response, whilst similar stimulation applied to the pituitary stalk or to the pituitary gland directly is ineffective. For this reason, and on the basis of the anatomical arrangements of the structures involved, it is likely that the stimulus from the hypothalamus is transmitted through the pituitary stalk, but by structures that are not excitable to electrical stimulation. The component of the stalk which appears to fulfil these requirements is the hypophysial portal vessels, and from the above results it seems likely that stimulation of the hypothalamus excites nerve fibres which transmit the stimulus to the pituitary gland via these vessels of the stalk."

From this "neuro-humoral" hypothesis regarding the control of the anterior pituitary gland by the hypothalamus via the portal vessels has sprung a vast body of scientific observations.

2.4. Electrical Stimulation Techniques

Stimulators

The early use of galvanic and faradic currents for stimulation

has now been superseded by the use of electronic stimulators in which the different parameters may be independently controlled with accuracy. This represents a great advance over older stimulation equipment which could only produce a constant voltage stimulus. If the aim of electrical stimulation is to present to the neural area under investigation a constant transfer of electrons from cathode to anode (and hence the production of depolarisation and action potentials in nerve cells) then such a constant flow demands a constant amperage. In constant voltage stimulation it can be seen from Ohm's law where voltage (V) in this case is a constant (k), that

$$V = k = IR,$$

where I is the current amperage and R the resistance in the circuit. Now if the circuit has a stable resistance, the amperage is also stable. In practice, in electrical stimulation circuits, the circuit resistance is unlikely to be stable. Cell death in the region of the electrode tracks; migration of tissue fluid ions; heating effects; reaction, if any, of electrode materials with tissue; deposition, if any, of ions from metallic electrodes; and evolution, if any, of gaseous material at electrodes at stimulation sites may all result in changes in circuit resistance. In constant voltage stimulation experiments, the choice of voltage levels has often been based apparently on the investigators preference. It is not uncommon to read that voltages above a certain level were not used because they were thought to be unphysiological.

This is palpably a nonsense. As Mickle (1961) puts it:

"It is worth noting that the impression of a single millivolt between two electrodes in or on the brain is also "unphysiological", and that once this neuronal disturbance has been introduced, few or no data exist which set an "unphysiological" limit to any particular electromotive (force) level".

Indeed, voltage level alone, regardless of how "physiologically" low it may be thought to be is meaningless unless some expression of the current flow is included at the same time. Mere expression of voltage is little indication of the energy dissipated in the tissue.

These disadvantages of constant voltage stimulators can be overcome in two ways. The first is imprecise, the second tedious. If one inserts in series in the stimulation circuit a very large resistor, then by Kirchhoff's Laws the total resistance in the circuit is equal to the sum of the separate resistances. That is, if the resistance in the preparation is Rp and the large resistance in the stimulation circuit is Rc, then the total resistance R is given by

$$R = Rp + Rc,$$

and the current I, in the circuit, assuming constant voltage k is given by

$$I = \frac{k}{Rp + Rc} = \frac{k}{R} .$$

Now, if Rc is sufficiently large, the contribution made to R by a changing Rp will be relatively small (when $Rc >> Rp$) and the

current will vary only slightly as Rp changes. However, the inclusion of a large resistance in the circuit means that the applied voltage necessary to produce a given current in the stimulation circuit must be large, and even then, the circuit current may still vary, albeit slightly.

The second solution involves simultaneous monitoring of current and voltage in the stimulation circuit by meters or cathode ray oscilloscopes and continual adjustment of voltage to maintain a steady current in the circuit. This method too can only be an approximation to achievement of an unvarying current in the stimulating circuit.

The advent of transistorised, constant current stimulators obviates the difficulties outlined above. They produce constant current output over a wide range of electrode resistances. (In circuits with alternating current (a.c.), both resistance and reactance occur, the reactance corresponding to the resistance of the capacitor formed by the electrodes. For simplicity, the term resistance will be used for both a.c. and direct current (d.c.) circuits, the reactance of the former circuit being included in the term resistance). The stimulator used in the experiments to be described is the Constant Current Stimulator (Model 7150, Nuclear-Chicago, Illinois). It produces monophasic or symmetrical and unsymmetrical biphasic "square" waves; pulse amplitude, duration

and frequency being independently variable. The maximum load resistance for a 1mA current is 100 Kohms and the current regulation has an accuracy of ± 1 % for a 100 % change in load resistance up to 100 Kohms. Figure 2.1 is a diagram of the stimulating circuit used in the experiments to be described.

The current from the stimulator is passed through a circuit containing a sensitive microammeter (Model V-T2, Taylor, Maidenhead). An electrically driven cam makes and breaks the circuit at 30 second intervals to allow pauses between trains of stimuli. A cathode ray oscilloscope (Model S51B, Telequipment, London) is connected across a 1 Kohm resistor and produces a trace of the stimulus being applied providing a visual calibration check on the stimulus parameters. A further check is provided by the ammeter, registering the amperage when d.c. is applied and registering the null point during a.c. stimulation, provided that the pulse pairs are accurately balanced.

Waveforms and Parameters of Stimulation

In theory, electrical stimulation of a part of the brain is easily achieved. Wires, insulated except at their tips, or glass tubes full of electrolyte are inserted into the brain, and current is passed between them, or between an individual electrode and an indifferent electrode exterior to the brain. In practice, it is important to select appropriate stimulation parameters, since various

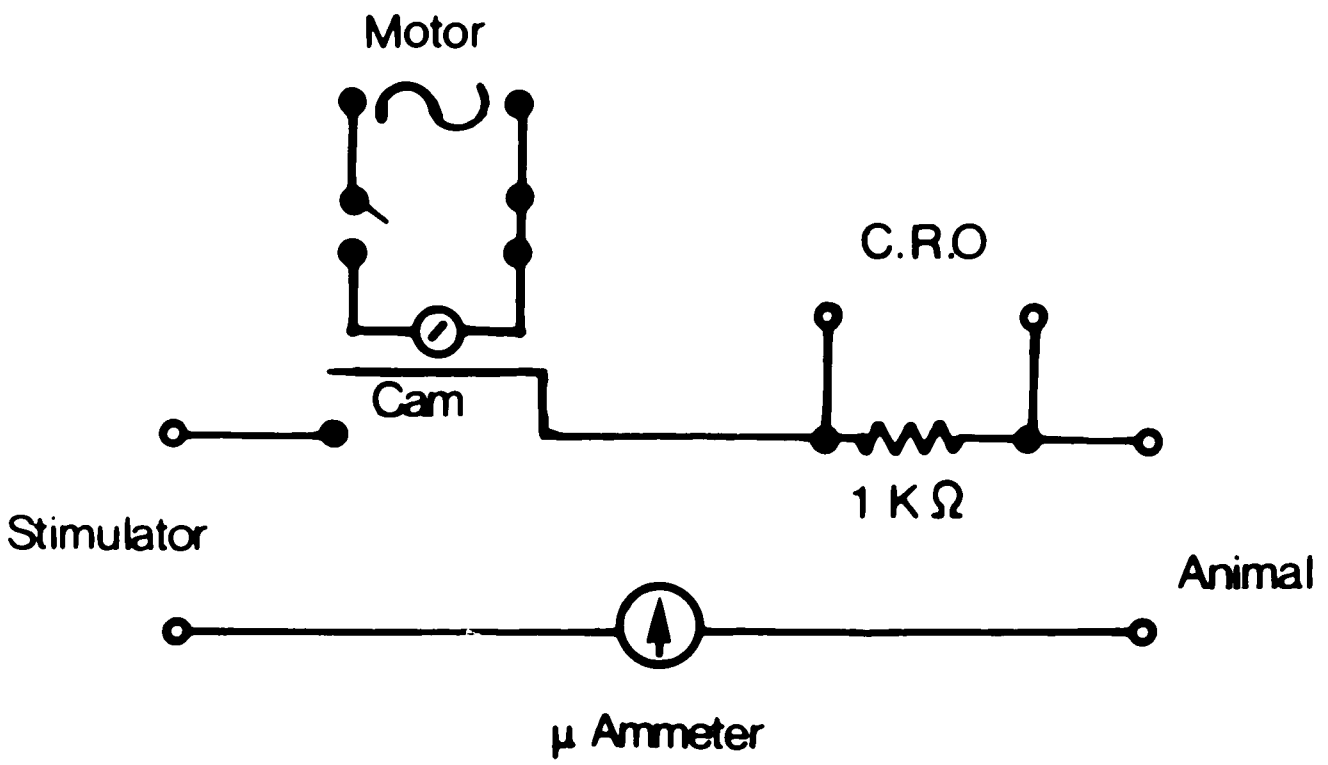


Figure 2.1 Diagram of circuit used in stimulation experiments.

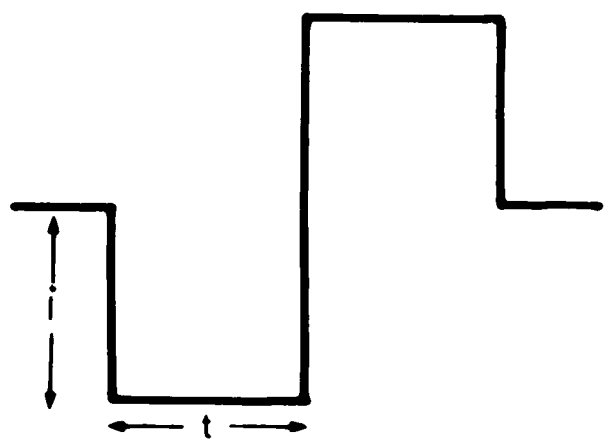


Figure 2.2 Diagram of a.c. stimulation waveform.

parts of the brain may not be equally excitable. In addition, it is important wherever possible to differentiate between a stimulating waveform, and one that will in fact cause a lesion in the area under examination, over and above that already caused by the insertion of the electrodes. The first emphasis, then, should be on avoidance of injury in stimulation experiments.

The injury caused to nervous tissue by insertion of electrodes is unavoidable, and will be termed the primary injury. It can be minimised by use of materials which have no toxic reaction with nervous tissue, and also by construction of electrodes with minimal dimensions (see Section 2.5).

The injury caused by passage of current will be termed the secondary injury. Two types of injury are possible. The passage of current, whether direct or alternating will cause thermal injury, the degree of injury being closely related to the total energy passed (Wattage) given by

$$\text{Wattage} = V \times I = I^2 R.$$

Assuming that the resistance across the electrodes remains constant, then the amount of heat developed by passage of the stimulating current will vary with the square of the current. Relatively small increases in current will therefore produce relatively large increases in heating. Heating effects will be

mitigated to some extent by conduction of heat away from the electrodes both directly through the tissue, and also by the blood stream in the vicinity of the electrodes.

The other type of secondary injury is electrolytic injury. The passage of current causes the movement of charged species in the electrical field set up by the potential difference between the electrodes. Products of electrolysis actually evolved at the electrodes may also damage surrounding nervous tissue. The amount of electrolytic injury will depend on the electrode materials (see Section 2.5) and also on the total quantity of undirectional electricity passed through the tissue in a given time (t) given by

$$Q = I \times t$$

where Q is the coulombage.

In this case, for d.c., it is essential to consider both amperage, and the time for which it is passed. For a.c., electrolytic injury is mitigated to some extent by the phase reversal, provided that the unidirectional pulse durations are relatively brief. If they are long, then clearly electrolytic injury may occur to such an extent with each undirectional pulse that the consequent reversed phase cannot hope to mitigate the injury.

It would seem appropriate therefore, to use a.c., of low amperage, for brief durations, thereby minimising heating and electrolytic injury. The waveform employed for a.c. stimulation in the experiments to

be described is a biphasic square wave (Figure 2.2). The height of each pulse (amperage) and the width (duration) are independently variable. This form of stimulation was introduced by Lilly, Hughes, Alvord and Galkin in 1955, for use in stimulation of the cerebral cortex of monkeys.

In experiments involving direct current stimulation, current was passed between the electrodes such that the intracranial electrode was positive with respect to the indifferent (rectal) electrode. With respect to d.c. stimulation, it is important to remember that Horsley and Clarke (1908) demonstrated that d.c. causes little excitation if increased from, and then decreased to, zero value sufficiently slowly to allow "accommodation" to take place in the nervous tissue. In addition, high frequency currents (greater than, say 200 KHz) can cause thermal injury by overheating the tissue, without excitation and without electrolytic injury if no rectification is allowed to occur in the circuit, and especially at the electrodes (Lilly, 1961).

To further complicate the interpretation of the sequelae of electrical stimulation of neural structures, it should be noted that once current of any type is passed, a wide variety of cells, which, if physiologically interconnected normally fire in sequence, now fire simultaneously so that there can be a concurrent excitation of excitatory and inhibitory neurones.

In conclusion, it should be realised that the stimulation of

very discrete areas of the brain may technically be very difficult, not only because of electrode localisation inaccuracies, but also because, depending on the parameters of stimulation employed, there may be spread of the stimulatory effect not only by direct conduction through surrounding nervous tissue (i.e. the electrical field set up round stimulating electrodes may embrace a larger volume of nervous tissue than is supposed) but also, by "leakage" through low resistance pathways (such as discrete nerve fibre bundles) to other areas of the brain. This second "spread" difficulty is a particular problem when indifferent electrodes outside the nervous system are employed.

Electrode Materials

The foundation of studies employing electrodes for electrical stimulation or lesioning of the brain was laid by Horsley and Clarke (1908) who studied cerebellar function. They employed unipolar electrodes of platinum-iridium wire insulated with glass capillary tubing, with the indifferent electrode located outside the cranial cavity. Many workers have constructed electrodes of stainless steel insulated with perspex or varnish in view of their robust nature and ease of manufacture. Glass-insulated platinum-wire electrodes are fragile and because of the glass insulation are often larger in diameter than steel electrodes. However, electrolysis in polar solutions employing stainless steel, nichrome, tungsten or silver electrodes leads to the liberation at the anode of the appropriate

metal ions. The high position of platinum in the electro-chemical series however, means that platinum ions are never released at the anode. It was not surprising then, that Loucks, Weinberg and Smith (1959) described the "erosion" of stainless steel, tungsten or silver electrodes after the passage of direct or alternating current in brain, and pointed out that results obtained in experiments with electrodes of these metals may be necessarily variable. Platinum-iridium electrodes (iridium neighbours platinum in the electro-chemical series) did not undergo erosion. The consequences of deposition of metal ions when steel electrodes are employed for lesion making has been appreciated only recently, despite the fact that Hillarp, Olivecrona and Silfverskiöld (1954) noted that lesions in the preoptic area made with steel electrodes evoked mating behaviour. Because this mating response was shortlived it was suggested that stimulation of nervous structures in the vicinity of the lesion may have occurred. Rowland and MacIntyre (1961) reported that deposition of iron occurred in an area much larger than the central area of histologically evident tissue destruction when the brain was lesioned with steel electrodes. Everett and his colleagues (Everett and Radford, 1961; Everett, 1964, 1965; Everett, Radford and Holsinger, 1964) found that ferric ions, deposited from stainless steel electrodes when lesions were made in the preoptic area of rats, caused ovulation.

Indeed the microinjection of 5 % ferric chloride solution alone was able to cause ovulation. Lesions of similar size in this area

produced by mechanical damage, radiofrequency current thermocautery, or electrolysis using platinum electrodes did not produce ovulation. It appears that metallic ions, such as ferrous and ferric ions, deposited in the brain produce a spherical zone of mild inflammation characterised by loss of neuronal cell bodies, gliosis, leucocytic infiltration and loss of the staining characteristics of fine unmyelinated fibres. At the margins of this zone, "chemical stimulation" of surrounding areas may persist for some time after the passage of current has ceased.

Donovan (1966b) comments that

"the use of steel electrodes in many studies of the effects of hypothalamic lesions means that the results may need re-interpretation, although the stimulative effects are likely to disappear and a depressive response predominate over the time course of chronic experiments." As evidence he (Donovan, 1966a) points out that lesions in the anterior hypothalamus are known to cause persistent oestrus in rats whether steel or platinum electrodes are used. This lesion effect lasts far longer than would be expected if it were due to "irritation" of the nervous tissue at the borders of the lesion. Surely the converse statement is of equal moment. Attention should be paid to electrode material choice in stimulation experiments as the stimulatory effect may be longer lasting when steel electrodes are used.

Sites of Stimulation

A diffuse neuronal system in the rat fore-brain occupying the septum pellucidum, medial preoptic and anterior hypothalamic areas has been postulated as an important component of the neural mechanism regulating the ovulatory discharge of gonadotrophins. This system is conceived to originate widely throughout the septal complex, to converge somewhat as it traverses the preoptic and anterior hypothalamic areas, and to move sharply to a basal location as it enters the tuberal region. Electrical stimulation of the medial preoptic area of Nembutal blocked pro-oestrous rats readily induces ovulation (Critchlow, 1958; Barraclough and Gorski, 1961; Everett and Radford, 1961; Barraclough, 1966; Terasawa & Sawyer, 1969), although the patterns and concentrations of follicle-stimulating hormone and luteinising hormone released into the circulation do not necessarily duplicate the normal pattern of release observed in unanaesthetised animals (Clemens, Shaar, Kleber & Tandy, 1971; Cramer & Barraclough, 1971; Kalra, Ajika, Krulich, Fawcett, Quijada & McCann, 1971).

Although many studies have been performed over the years on the activation of LH release (or ovulation) in female rats by electrical stimulation of the hypothalamus, there are few reports of this phenomenon in males. Moll and Zeilmaker (1966) employed unipolar steel electrodes implanted either into the median eminence or into the medial preoptic area of castrated male rats bearing isologous ovarian grafts under the kidney capsule. Castration and

transplantation occurred at 10 weeks of age, and stimulation 2 - 6 weeks later. Using d.c. stimulation of 1.5 mA for 10 seconds they demonstrated follicles containing ova with dispersed cumulus oophorus cells. Many of the follicles showed early luteinisation changes, whereas sham stimulated animals showed only follicles in various stages of development and no corpora lutea. They were unable to distinguish between ovarian changes caused by median eminence stimulation and those caused by medial preoptic area stimulation. Similar results were obtained by Quinn (1966) who used much smaller stimulatory currents (10 μA d.c. for 20, 40 or 60 sec.) delivered through concentric bipolar stainless steel electrodes implanted in the medial preoptic area.

In the experiments to be described, electrodes were implanted into the medial preoptic area (or on occasions into the anterior hypothalamic area) of normal male rats; a variety of electrode materials and parameters of stimulation being employed.

2.5. Experimental Methods

Animals

Experimental animals were adult male and female rats of the Wistar strain (Charles River (U.K.) Ltd.; Margate) weighing 220 - 290 g. All animals were maintained under conditions of controlled light (14 h. light, 10 h. dark) and temperature (22° C) and fed

diet 41 B pellets (E. Dixon & Sons Ltd.; Ware; Appendix 2.1) and tap water ad libitum.

Electrode Manufacture

In the experiments to be described, both platinum and steel electrodes were used. Platinum electrodes were constructed from platinum wire (Precious Metals Ltd.; London) 0.125 mm in diameter. Glass capillary tubing was drawn to an inside diameter sufficient to tightly enclose the wire and fixed in place with cement (Araldite, Ciba-Geigy; Cambridge). Insulated copper wire was soldered to the free end of the platinum wire and the whole assembly was mounted in a teflon jig and fixed in place with acrylic cement (Dental Fillings Ltd.; London). Both single and twin unipolar electrodes were constructed. In all cases electrodes were 13 mm in length with a bared tip 0.3 mm in length. Twin unipolar electrodes were parallel and separated by 2.0 mm. In the insulated part the electrode diameter was 0.22 mm.

Steel electrodes were constructed from stainless steel micropins (Watkins and Doncaster; Kent) 0.125 mm in diameter insulated with glass as above. Some steel electrodes were insulated with lacquer (Bakelite Ltd.; Birmingham) baked at 60° C for 6 hours. Lacquer was scraped off to provide a bared tip 0.3 mm in length. In other respects, these steel electrodes were similar to platinum electrodes. Final diameter in the lacquer insulated part was 0.18 mm.

The assistance of Mr. R. Corney, Miss S. Chiappa and Miss V. Langford with electrode making is gratefully acknowledged.

Electrode Insertion

Animals were anaesthetised with 10 % ethyl carbamate (Urethane, BDH; Poole) in 0.9 % saline by intraperitoneal injection at a dose of 10 ml/kg. After insertion into a stereotaxic apparatus (made by Mr. R. Corney, Department of Human Anatomy, Oxford University) calibrated for use with the stereotaxic atlas of the rat fore-brain of de Groot (1959), the scalp was incised in the midline. The cranial periosteum was elevated to expose the dorsal surfaces of the frontal and parietal bones and a dental machine (Kaltenbach & Voss; W. Germany) used to drill holes in the cranium as appropriate. Bone fragments were removed, taking care to avoid compression of the cerebral hemispheres. The cranial endosteum and the dura mater were punctured to facilitate electrode entry taking care to avoid laceration of the superior sagittal sinus. Bleeding was controlled by light pressure with cotton wool pledgets. The electrode was then lowered into position using a triplanar micromanipulator (Prior; Bishop Stortford, Herts.) equipped with millimeter vernier gauges.

Electrode Site Verification

According to the stereotaxic atlas of the rat forebrain (de Groot, 1959) designed for rats of 200 - 300 g body weight, the

preoptic area lies 8 mm anterior to the vertical zero plane passing through the interaural line, and 1.5 mm below the horizontal zero plane. The horizontal zero plane is situated 5.0 mm above the centre of the interaural line, when the incisor bar supports the upper jaw at a rostral reference point located immediately behind the upper incisor teeth at a height of 5.0 mm above the level of the interaural line. The equivalent co-ordinates for the anterior hypothalamic area are 6.8 mm anterior to the vertical zero plane, and 2.0 mm below the horizontal zero plane. Electrodes implanted according to these co-ordinates were visualised on lateral radiographs using a Practix (Philips; Holland) X-ray machine and Kodirex film (Kodak; London). The electrode tips (Figure 2.3) can be seen to lie superior to the basisphenoid bone and slightly anterior to the shadow of the squamosal, when implanted into the medial preoptic area. In the original radiographs it is usually possible to see the presphenoido-basisphenoid suture. Correctly placed electrodes lie 1.0 to 1.5 mm above this suture.

At the end of experiments the electrodes were withdrawn, and the head fixed in 10 % buffered formalin at pH 7.0. The brain was dissected out of the cranium and coronal sections cut on a freezing microtome (Leitz; Germany) at 40 μm. Haematoxylin and eosin, thionin or neutral red (Gurr; London) stained serial sections were examined for electrode tracks and their terminations (Figure 2.4).
A diagram of electrode sites appears in Appendix 2.4 On

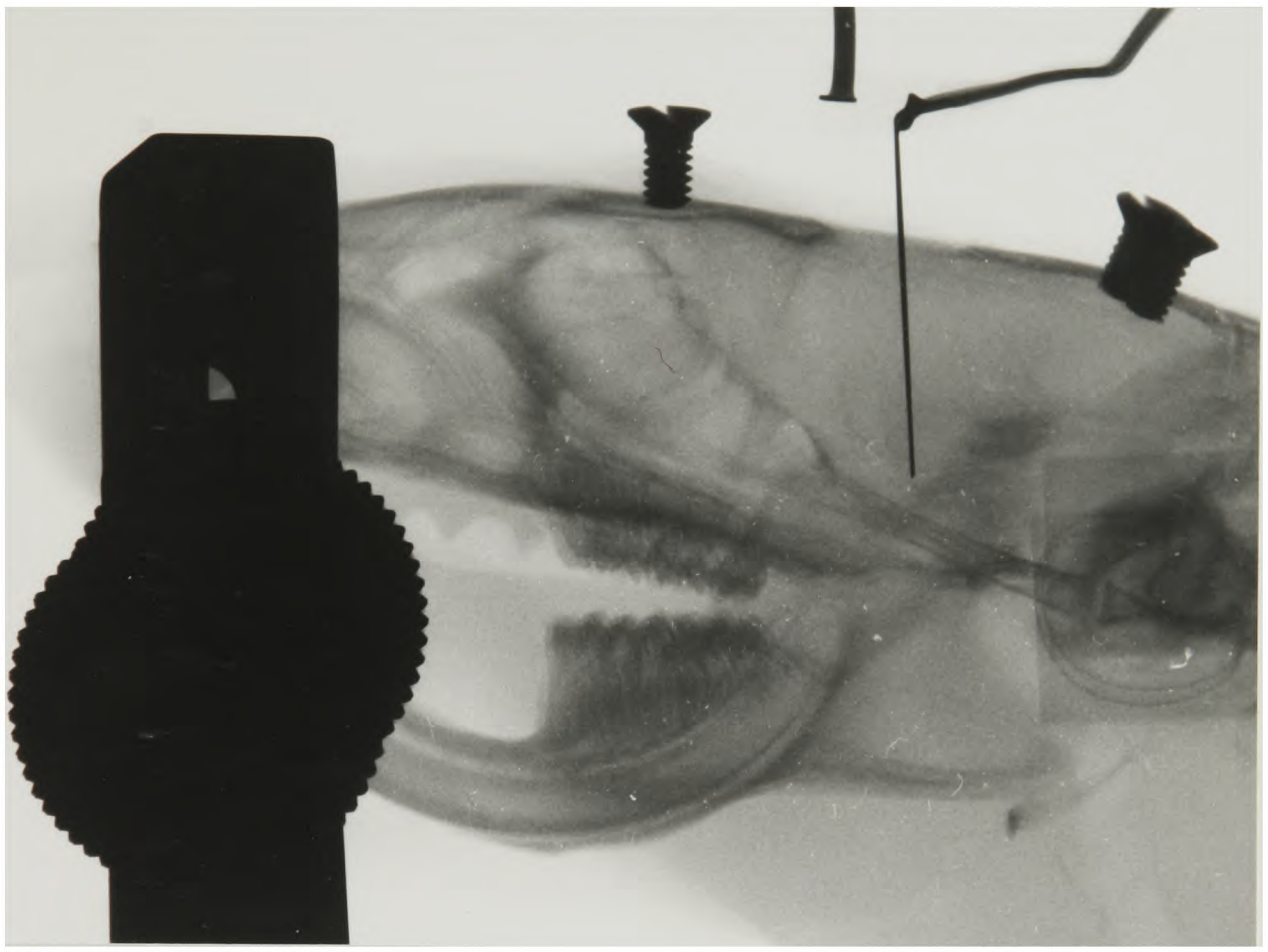


Figure 2.3

Plate. Lateral radiograph of electrodes inserted into the medial preoptic area.

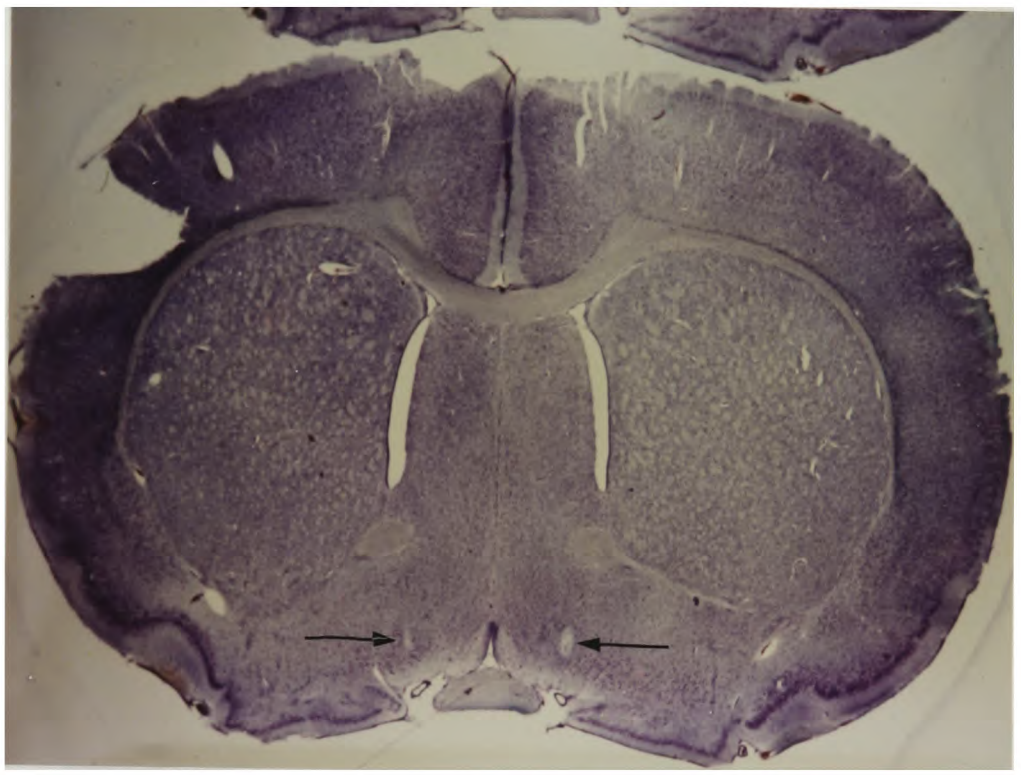


Figure 2.4

Plate. Photomicrograph of thionin stained coronal section of rat brain. Electrode terminations (↑) lie in the medial preoptic area (a.c. stimulation).

occasions when steel electrodes were used, sections were treated with 2 % potassium ferrocyanide solution in formol-saline to ascertain the presence of iron at electrode sites (see Section 2.9).

Blood Collection

With the animal fixed in the stereotaxic machine, the left femoral vein was exposed by an incision, dissected free of the femoral artery and ~~vein~~ nerve and cannulated with Portex flexible nylon tubing, size 00 (Portland Plastics Ltd.; Kent). The cannula was connected to a three way stopcock (Pharmaseal Lab.; U.S.A.). Samples of blood, 0.5 ml in volume were withdrawn immediately prior to passage of current, and thereafter at 5, 10, 20, 40, 60 and 80 minutes. After each withdrawal, 0.5 ml of 0.9 % saline containing 100 IU heparin/ml (Pularin, Evans Medical Ltd.; Liverpool) was injected through the cannula.

Blood samples were transferred to ice-cooled centrifuge tubes, centrifuged (Minor, MSE; London) to separate cells from plasma, and the plasma was stored at -15° C until assay.

Assay of Luteinising Hormone

The luteinising hormone (LH) content of plasma was measured by the ovine-ovine double antibody radioimmunoassay (RIA) method developed by Niswender, Midgley, Monroe & Reichert (1968) and the

results expressed as ng/ml plasma of the ovine standard (NIH - LH - S 13) used in the assay. All samples were assayed in duplicates of 50 µl plasma/assay tube. The lower limit of sensitivity of the assay for 50 ul samples was 1 ng/ml in terms of the LH standard, the antiserum used being GDN # 15.

Assay Protocol

The assay is set up and all manipulations are carried out at 4° C in an ice bath.

Day I

(i) Samples (50 - 200 µl) and Standards (200 µl). Add 0.01 M phosphate buffered saline/1 % egg white such that final volume is 400 µl.

(ii) Blanks. Add 400 µl PBS/EW.

(iii) Total Bound. Add 400 µl PBS/EW.

(iv) To sample, standard and total bound tubes add 200 µl antiserum diluted to 1:60,000 with 0.01 M PBS/0.05 M EDTA/1:300 NRS.

(v) To blank tubes add 200 µl PBS/EDTA/NRS.

(vi) Whirlimix and incubate at 4° C. for 24 hrs.

Day II

(i) To all tubes, (including Total Count tubes) add 200 µl of ^{125}I labelled LH (LER 1056 C-2) containing 10,000 counts/minute, diluted in PBS/EW. (Counter efficiency 35%)

(ii) Whirlimix, and incubate at 4° C for 48 hrs.

Day IV

(i) To all tubes (excluding Total Count tubes) add 200 µl of anti-rabbit gamma-globulin diluted to 1:30 with 0.01 M PBS.

(ii) Whirlimix, and incubate at 4° C for 24 hrs.

Day V

(i) Centrifuge for 40 minutes at 3000 r.p.m. at 4° C.

(ii) Aspirate supernatant.

(iii) Count all tubes for 10 min. or 10,000 counts.

Assay of Follicle-Stimulating Hormone

The follicle-stimulating hormone (FSH) content of plasma was measured with the NIAMD rat FSH radioimmunoassay kit. Results are expressed in terms of ng/ml NIAMD - rat FSH - RP - 1. In the majority of samples insufficient plasma was available to permit estimations in duplicate. These samples were assayed using single 100 µl quantities. The lower limit of sensitivity for 100 µl samples was 75 ng/ml in terms of the FSH standard, the antiserum used being NIAMD - antirat - FSH - S6.

Assay Protocol

The assay is set up and all manipulations are carried out at 4° C in an ice bath.

Day I

(i) Samples (50 - 200 µl) and Standards (200 µl). Add 0.01 M phosphate buffered saline/1.0 % egg white such that the final volume is 400 µl.

(ii) Blanks. Add 400 µl PBS/EW.

(iii) Total Bound. Add 400 µl PBS/EW.

(iv) To sample, standard and total bound tubes add 200 µl antiserum diluted to 1:2500 with 0.01 M PBS/0.05 M EDTA/1:300 NRS.

(v) To blank tubes add 200 µl PBS/EDTA/NRS.

(vi) To all tubes (including Total Count tubes) add 200 µl of labelled FSH, (NIAMD - rat - FSH - 1 - 1) containing 12,000 c.p.m., diluted in PBS/EW. (Counting efficiency 35%)

(vii) Whirlimix, and incubate at 4° C for 96 hrs.

Day V

(i) To all tubes (excluding Total Count tubes) add 200 µl of anti-rabbit gamma-globulin diluted to 1:30 with 0.01 M PBS.

(ii) Whirlimix, and incubate at 4° C for 24 hrs.

Day VI

(i) Centrifuge for 40 minutes at 3000 r.p.m. at 4° C.

(ii) Aspirate supernatant.

(iii) Count all tubes for 10 min. or 10,000 counts.

2.6. Stimulation Parameters - Frequency

In the past, the frequency used in electrical stimulation of the hypothalamus has varied widely. Clearly the total charge passed through the tissue will depend on the height and width of each pulse or pulse pair, as well as on the frequency. In the present experiments height and width of pulse pairs were kept constant while the frequency was varied. Frequencies of 15, 30, 60, 90 and 120 Hz were used. This range encompasses the vast majority of frequencies used in experiments in the past, from the 15 Hz used by Martin (1972) in his studies on plasma growth hormone response to hypothalamic stimulation, to the 100 Hz used by Cramer and Barraclough (1971, 1973) in their studies of plasma LH concentrations after hypothalamic stimulation. Other workers (Everett, 1965; Harris and Ruf, 1970; Meijs-Roeloffs, 1972) have used frequencies from 30 - 60 Hz.

Present Experiments

The results of varying frequencies of stimulation can be seen in Table 2.1 and Figure 2.5. Control animals which had electrodes inserted, but no current passed, show little variation in LH concentration over the time course of the experiments. In animals which were stimulated the waveform consisted of balanced biphasic square waves of 1 msec. duration and 0.5 mA. amplitude. Trains of stimuli were applied for 30 sec. followed by a pause of 30 sec.,

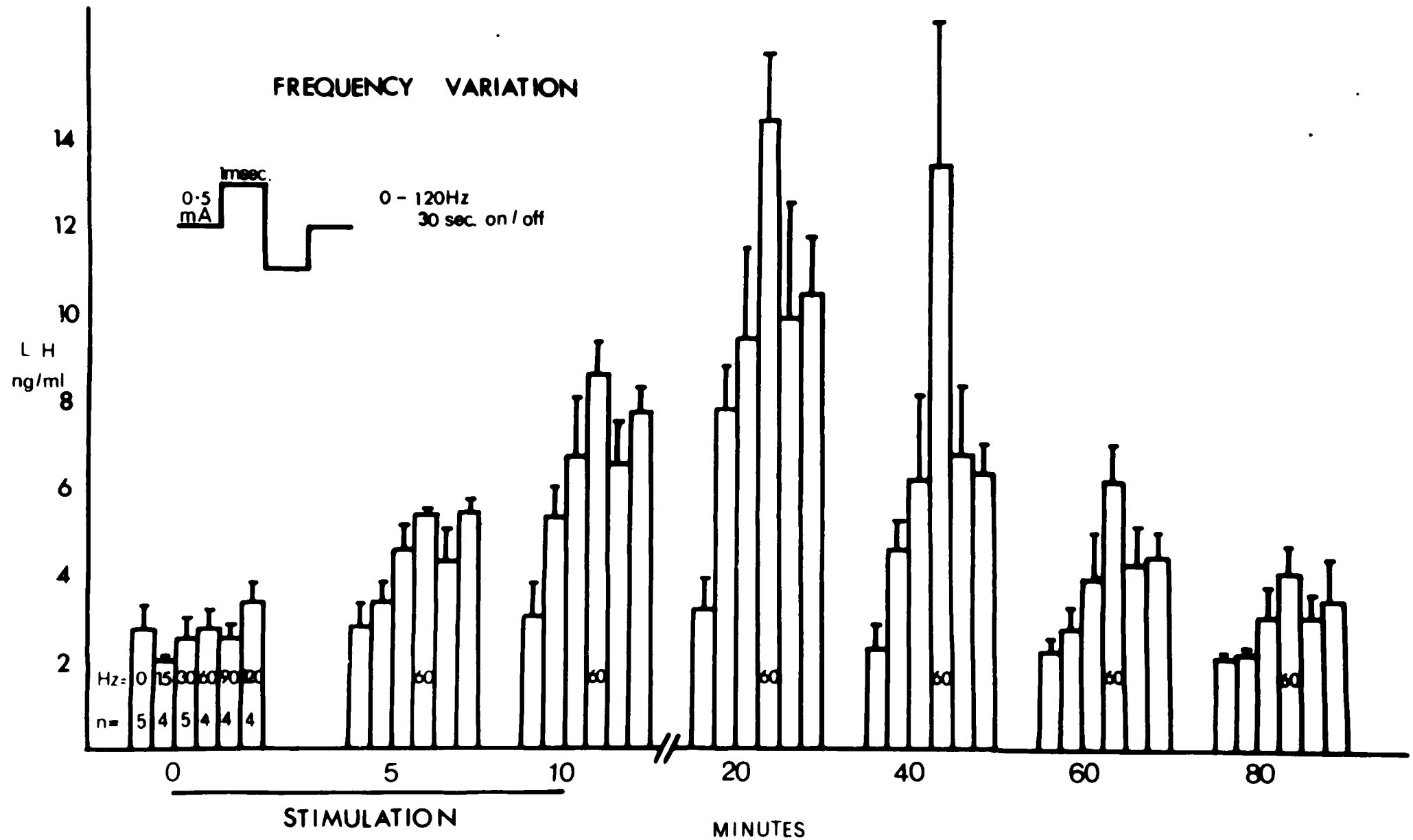
Table 2.1

Mean peripheral LH concentrations (± S.E.M. at various times during and after medial preoptic area biphasic stimulation at various frequencies with constant pulse duration (1 msec.) and amplitude (0.5 mA). 10 min. stimulation, 30 sec. on/off.

Time (min.)	Frequency (Hz)	0	15	30	120	90	60
	Q (mC)	0	4.5	9.0	36.0	27.0	18.0
	n	5	4	5	4	4	4
0		2.8 ± 0.5	2.0 ± 0.1	2.6 ± 0.4	3.4 ± 0.4	2.6 ± 0.3	2.8 ± 0.4
5		2.9 ± 0.5	3.4 ± 0.5	4.6 ± 0.7	5.4 ± 0.3	4.2 ± 0.8	5.4 ± 0.1
10		3.1 ± 0.8	5.4 ± 0.7	6.7 ± 1.6	7.7 ± 0.5	6.5 ± 1.4	8.6 ± 0.7
* 20		3.2 ± 0.7	7.8 ± 1.1	9.5 ± 2.3	10.5 ± 1.3	9.9 ± 2.7	14.5 ± 1.5
* 40		2.3 ± 0.5	4.5 ± 0.7	6.2 ± 2.0	6.3 ± 0.7	6.7 ± 1.5	13.4 ± 3.4
60		2.2 ± 0.3	2.7 ± 0.5	3.9 ± 1.1	4.4 ± 0.5	4.2 ± 0.8	6.1 ± 0.8
80		2.1 ± 0.1	2.1 ± 0.1	3.1 ± 1.0	3.4 ± 0.9	3.0 ± 0.5	4.0 ± 0.7

NOTE. * Any two means not underscored by the same solid (interrupted) line are significantly different at the 5 % (1 %) level.

Figure 2.5 Histogram showing peripheral plasma LH concentrations prior to, during, and after medial preoptic area a.c. stimulation at various frequencies (Platinum electrodes). In this and subsequent histograms vertical lines above bars denote S.E.M.



for a total of 10 min. Stimulation at all frequencies tested led to rises in LH concentration, maximal at 20 min. It appears that stimulation applied from 0 to 10 min. is most efficacious in causing the release of LH, as assessed by measurement made at 20 and 40 min. when applied at a frequency of 60 Hz. There is considerable variability of response to stimulation.

Differences between groups at 20 and 40 min. were tested by applying the Multiple Range Test of Duncan (1955) using the Critical Values for this test compiled by Harter (1960). While other sampling times 10 min., 60 min., and 80 min. gave similar but less marked patterns, it seemed unlikely that any significant difference between groups would be found, except in some cases between control values and stimulated values. The full work sheet for the calculations for these present results at 40 min. is appended in Appendix 2.2.

In the present experiments the peripheral LH concentration at 40 min. was significantly greater in animals stimulated at 60 Hz, than in those stimulated at 0 (control), 15, 30, 90 and 120 Hz ($p < 0.01$). At 20 min. this difference was not significant ($p > 0.05$).

It is noteworthy that the total coulombage applied in each group is not directly related to the response in peripheral LH concentration. At 120 Hz the coulombage applied (36.0 mC) is twice that applied at 60 Hz, yet the response is markedly smaller.

On the basis of these results it was decided to proceed with the examination of other parameters of stimulation, keeping the stimulation frequency constant at 60 Hz.

2.7. Stimulation Parameters - Pulse Amplitude

There has been much variation in current amperage used in electrical stimulation of the hypothalamus. The few workers who have used balanced biphasic pulse pairs in stimulation include Everett (1965) who delivered 0.5 mA/pulse, Cramer and Barraclough (1971, 1973) 0.14 mA/pulse, Harris and Ruf (1970) 0.5 mA/pulse, Martin (1972) 0.3 - 0.7 mA/pulse and Martin, Kontor and Mead (1973) 0.5 - 0.7 mA/pulse.

Present Experiments

In the experiments to be described the frequency and pulse duration were held constant at 60 Hz and 1 m sec. respectively, while the pulse amplitude was set at 0.05, 0.10, 0.25, 0.50 and 1.00 mA in successive groups. This range encompasses the amplitudes of stimulation generally employed in the past for hypothalamic stimulation.

The results of varying pulse amplitudes on peripheral LH concentration can be seen in Table 2.2 and Figure 2.6. Again, the stimulus was applied for 10 min, 30 sec on/off. Stimulation with pulses having amplitudes of 0.05 and 0.10 mA had no significant

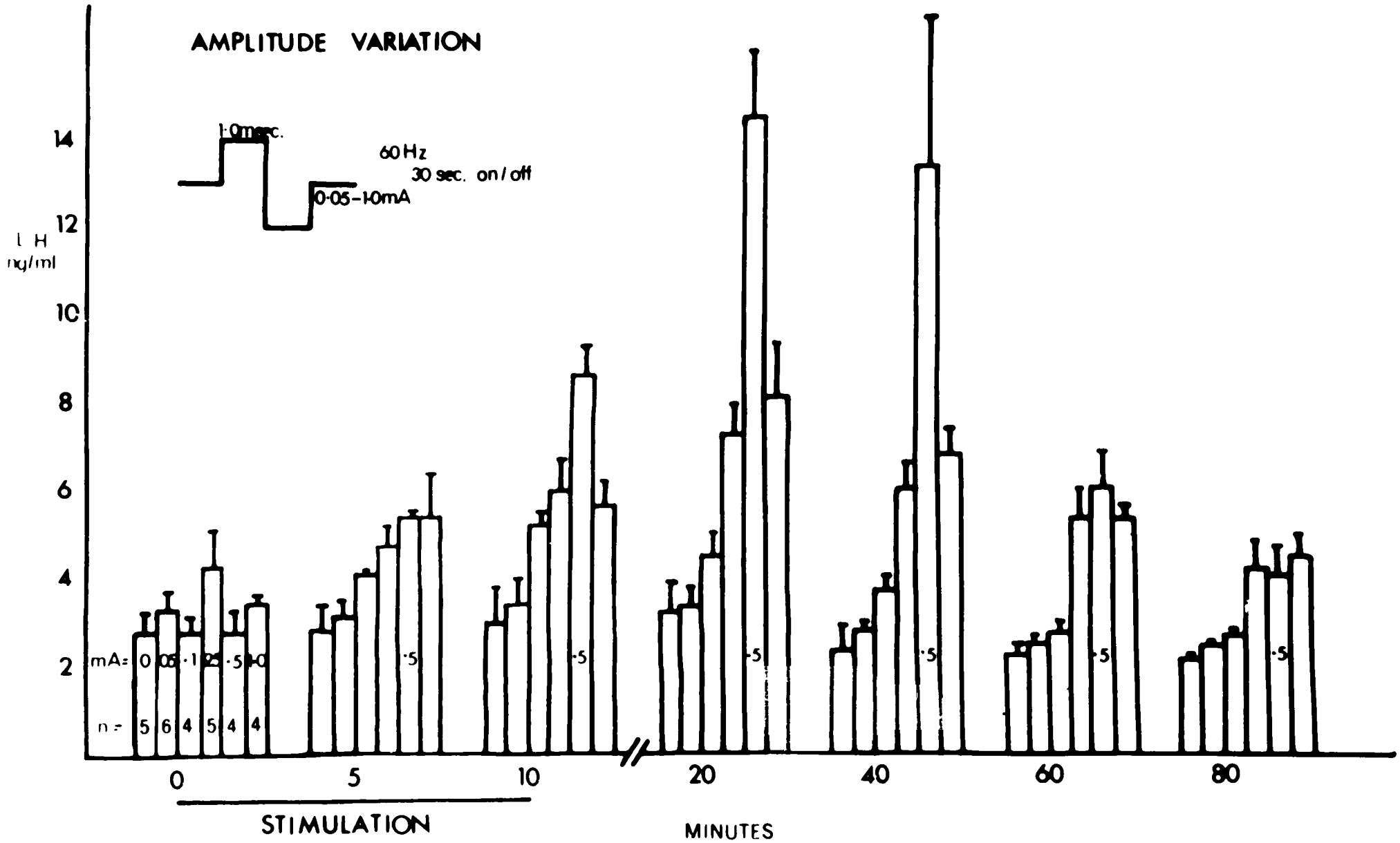
Table 2.2

Mean peripheral LH concentrations (± S.E.M.) at various times during and after medial preoptic area biphasic stimulation with varying pulse amplitude, and constant pulse duration (1 msec.) and frequency (60 Hz). 10 min. stimulation, 30 sec. on/off.

Time (min.)	Amplitude (mA)	0	0.05	0.10	0.25	1.0	0.5
	Q (mC)	0	1.8	3.6	9.0	36.0	18.0
	n	5	6	4	5	4	4
0		2.8 ± 0.5	3.3 ± 0.4	2.8 ± 0.3	4.3 ± 0.8	3.5 ± 0.2	2.8 ± 0.4
5		2.9 ± 0.5	3.1 ± 0.4	4.1 ± 0.1	4.7 ± 0.6	5.4 ± 1.0	5.4 ± 0.1
10		3.1 ± 0.8	3.4 ± 0.6	5.2 ± 0.3	6.0 ± 0.8	5.7 ± 0.6	8.6 ± 0.7
* 20		3.2 ± 0.7	3.3 ± 0.5	4.5 ± 0.6	7.3 ± 0.7	8.1 ± 1.3	14.5 ± 1.5
* 40		2.3 ± 0.5	2.8 ± 0.3	3.7 ± 0.3	6.0 ± 0.7	6.8 ± 0.7	13.4 ± 3.4
60		2.2 ± 0.3	2.6 ± 0.1	2.8 ± 0.2	5.3 ± 0.8	5.3 ± 0.3	6.1 ± 0.8
80		2.1 ± 0.1	2.4 ± 0.1	2.7 ± 0.1	4.3 ± 0.7	4.4 ± 0.5	4.0 ± 0.7

NOTE. * Any two means not underscored by the same solid (interrupted) line are significantly different at the 5 % (1 %) level.

Figure 2.6 Histogram showing peripheral plasma LH concentrations prior to, during, and after medial preoptic area a.c. stimulation at various pulse amplitudes (Platinum electrodes).



effect on peripheral LH concentration.

Amplitudes of 0.25, 1.00 and particularly 0.50 mA markedly increased peripheral LH concentration, particularly at 20 and 40 min. While considerable variability of response is evident, at these times the LH concentration was significantly greater in animals stimulated with pulses of amplitude 0.5 mA than in other groups ($p < 0.01$). Again there is no direct relation between total coulombage applied in each group and the response in peripheral LH concentration.

2.8. Stimulation Parameters - Pulse Duration

In the past investigators have generally used pulses 1 m sec. in length, although Martin, Kontor and Mead (1973) used pulses as short at 0.2 m sec. and as long as 2.0 m sec.

Present Experiments

In the experiments to be described the frequency and pulse amplitude were held constant at 60 Hz and 0.5 mA respectively, while the pulse duration was set at 0.25, 0.50, 1.00 and 2.00 m sec. in successive groups.

The results of varying pulse durations on peripheral LH concentrations can be seen in Table 2.3 and Figure 2.7. Stimuli with pulse durations from 0.25 to 2.0 m sec. markedly increased

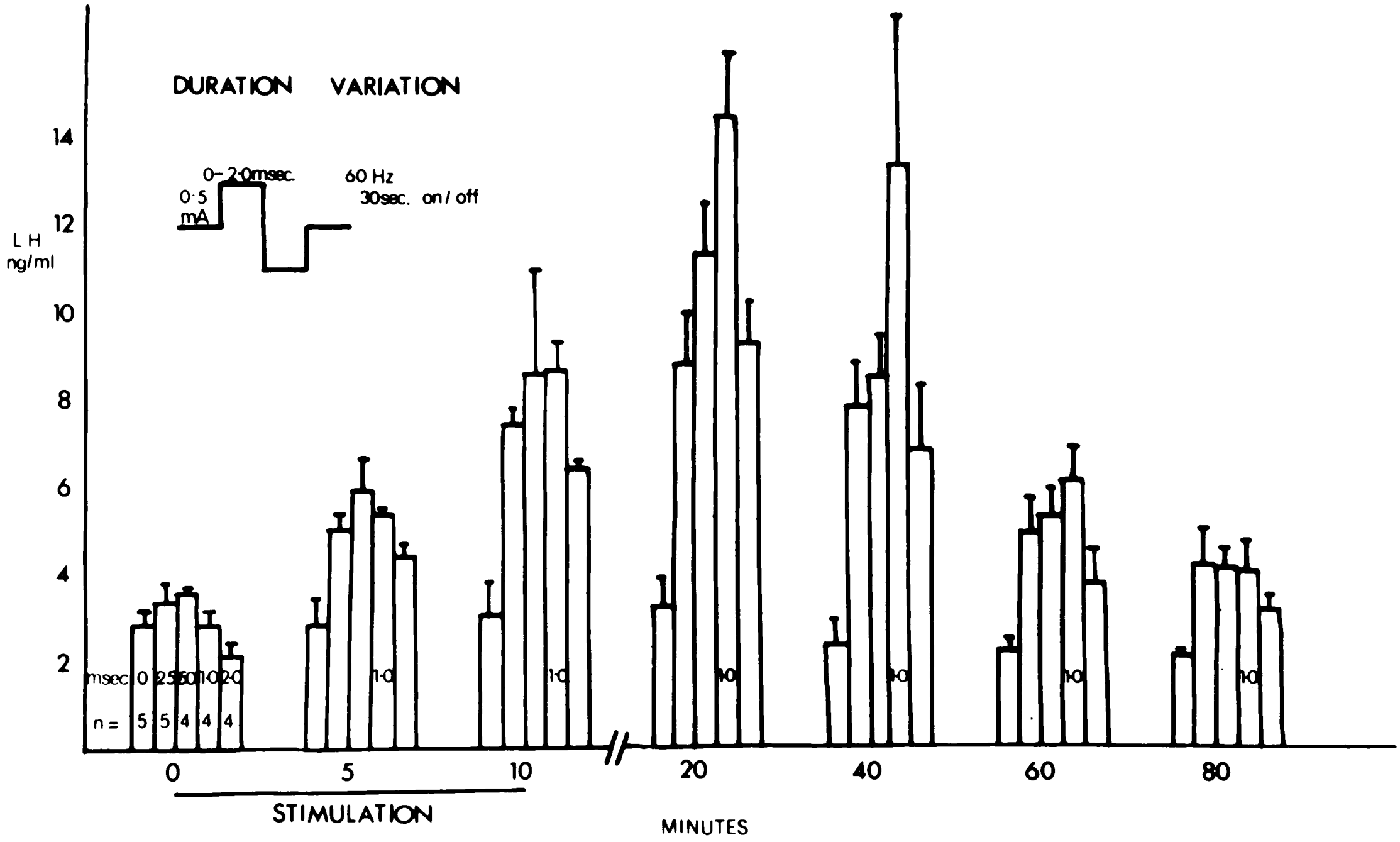
Table 2.3

Mean peripheral LH concentrations (± S.E.M.) at various times during and after medial preoptic area biphasic stimulation with varying pulse duration, and constant pulse amplitude (0.5 mA) and frequency (60 Hz). 10 min. stimulation 30 sec. on/off.

Time (min.)	Duration (msec.)	0	0.25	2.0	0.5	1.0
	Q (mC)	0	4.5	36.0	9.0	18.0
	n	5	5	4	4	4
0		2.8 ± 0.5	3.4 ± 0.5	2.1 ± 0.2	3.5 ± 0.1	2.8 ± 0.4
5		2.9 ± 0.5	5.0 ± 0.4	4.4 ± 0.3	5.9 ± 0.8	5.4 ± 0.1
10		3.1 ± 0.8	7.4 ± 0.4	6.5 ± 0.2	8.5 ± 2.6	8.6 ± 0.7
* 20		3.2 ± 0.7	8.8 ± 1.2	9.3 ± 1.0	11.3 ± 1.5	14.5 ± 1.5
* 40		2.3 ± 0.5	7.8 ± 1.0	6.8 ± 1.5	8.5 ± 1.1	13.4 ± 3.4
60		2.2 ± 0.3	4.9 ± 0.8	3.7 ± 0.8	5.3 ± 0.6	6.1 ± 0.8
80		2.1 ± 0.1	4.2 ± 0.8	3.1 ± 0.3	4.0 ± 0.5	4.0 ± 0.7

NOTE. * Any two means not underscored by the same solid (interrupted) line are significantly different at the 5 % (1 %) level.

Figure 2.7 Histogram showing peripheral plasma LH concentrations prior to, during, and after medial preoptic area a.c. stimulation at various pulse durations (Platinum electrodes).



peripheral LH concentration. At 20 and 40 min., when this increase was greatest, it appeared that stimuli with a pulse duration of 1.0 m sec. were most effective. However, it can be seen from Table 2.3 that there was no significant difference between peripheral LH concentrations produced by stimuli with pulse durations of 1.0 and 0.5 m sec. Pulse durations of 1.0 and 0.5 m sec. were, however, more effective than pulse durations of 0.25 and 2.0 m sec. at 20 mins. ($p < 0.05$) and 40 min. ($p < 0.05$).

2.9. Stimulation Parameters - Electrode Materials

Reference has been made to the problem of metallic electrode materials in a previous section. Experiments were carried out to compare platinum and steel electrodes constructed to the same dimensions, and insulated with glass. The electrodes were implanted acutely, and electrical stimulation carried out with optimal parameters as determined in the preceding three sections for a ten minute period, 30 sec. on/off.

Present Experiments

The results of these experiments appear in Table 2.4 and Figure 2.8. The results for "platinum control" and "platinum stimulation" groups have been given in previous sections. The peripheral LH concentration in animals stimulated with platinum electrodes returns to initial levels by 80 min.

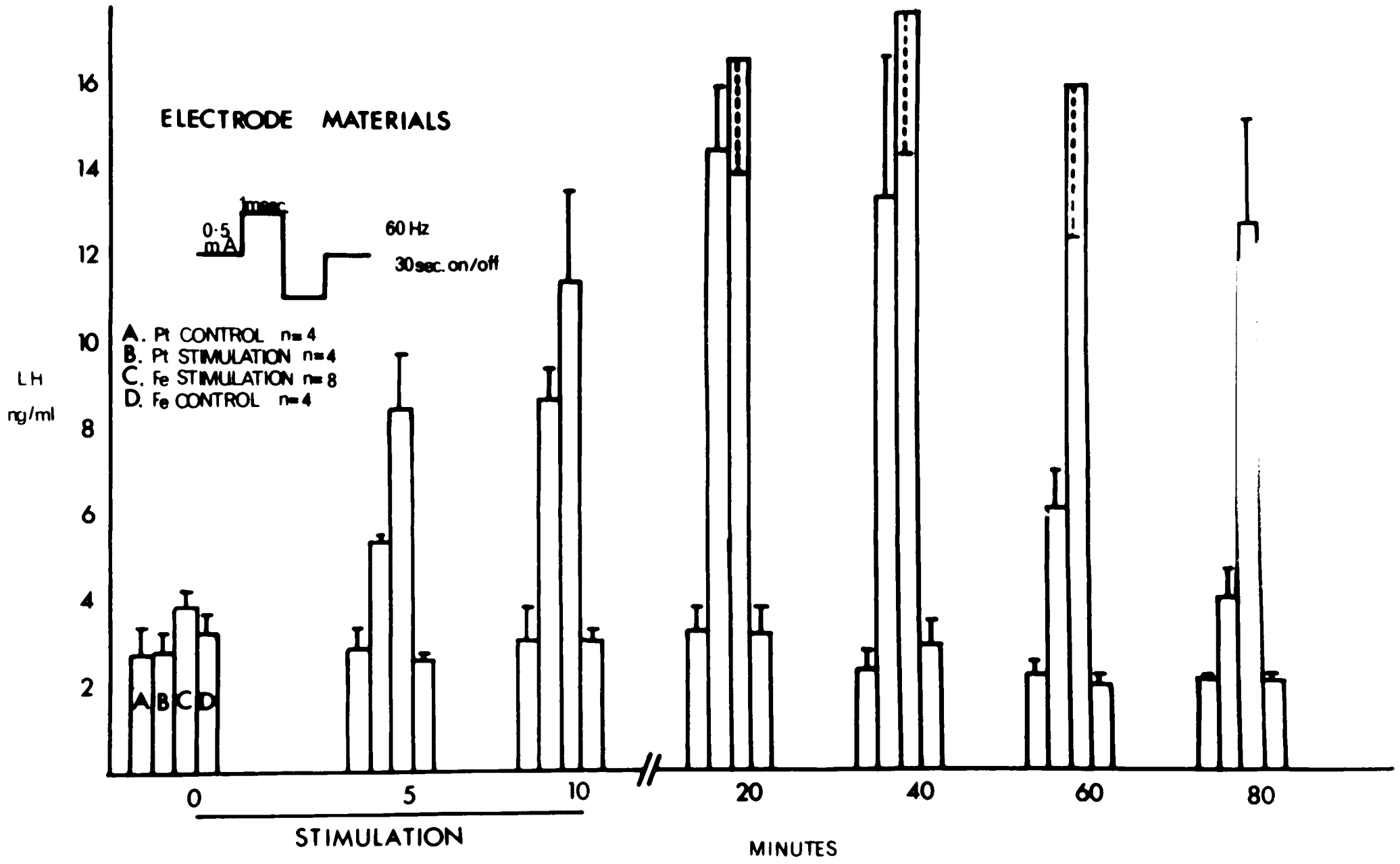
Table 2.4

Mean peripheral LH concentrations (± S.E.M.) at various times during and after medial preoptic area biphasic stimulation at frequency 60 Hz, pulse duration 1.0 msec., pulse amplitude 0.5 mA with platinum and with steel electrodes. 10 min. stimulation 30 sec. on/off.

Time (min.)		Pt Control	Pt Stimulation	Fe Stimulation	Fe Control
	Q (mC)	0	18.0	18.0	0
	n	4	4	8	4
0		2.8 ± 0.5	2.8 ± 0.4	3.8 ± 0.4	3.2 ± 0.4
5		2.9 ± 0.5	5.4 ± 0.1	8.4 ± 1.3	2.6 ± 0.1
		* p< 0.01		* p< 0.02	
10		3.1 ± 0.8	8.6 ± 0.7	11.4 ± 2.1	3.0 ± 0.3
		* p< 0.01		* p< 0.05	
20		3.2 ± 0.7	14.5 ± 1.5	16.6 ± 2.6	3.2 ± 0.6
		* p< 0.001		* p< 0.01	
40		2.3 ± 0.5	13.4 ± 3.4	18.0 ± 3.6	2.9 ± 0.6
		* p< 0.02		* p< 0.02	
60		2.2 ± 0.3	6.1 ± 0.8	15.9 ± 3.5	2.5 ± 0.2
		* p< 0.01		* p< 0.05	
80		2.1 ± 0.1	4.0 ± 0.7	12.8 ± 2.4	2.0 ± 0.2
		* p< 0.05	* p< 0.05	* p< 0.02	

* Students t test.

Figure 2.8 Histogram showing peripheral plasma LH concentrations prior to, during, and after medial preoptic area a.c. stimulation with either platinum or steel electrodes.



With steel electrodes a similar response pattern emerges up to 40 min. Thereafter the "steel stimulated" LH levels remain elevated and up to the 80 min. sampling period fall far less quickly. There is considerable variability of response in steel stimulated animals, and only at 80 min. is there a significant difference in LH concentration between steel and platinum stimulated groups ($p < 0.05$).

In order to determine whether metal ions had been deposited during the period of a.c. stimulation, sections of these brains were treated with 2 % potassium ferrocyanide solution. Figure 2.9 is a photomicrograph of such a section. It can be seen that there has been deposition of metal ions in the region of both of the electrode tips. No such deposits could be detected in "steel control" sections. Evidently, simple contact between steel electrodes and brain substance over the period of blood sampling does not lead to significant changes in peripheral LH concentration.

2.10. Stimulation Parameters - Direct Current

In 1961 Everett and Radford demonstrated clearly that the passage of d.c. through steel electrodes inserted into the preoptic area was capable of causing ovulation in rats, whereas the passage of the same current through platinum electrodes had no such ovulatory effect. They applied 15 μA d.c. for 10 sec. through concentric

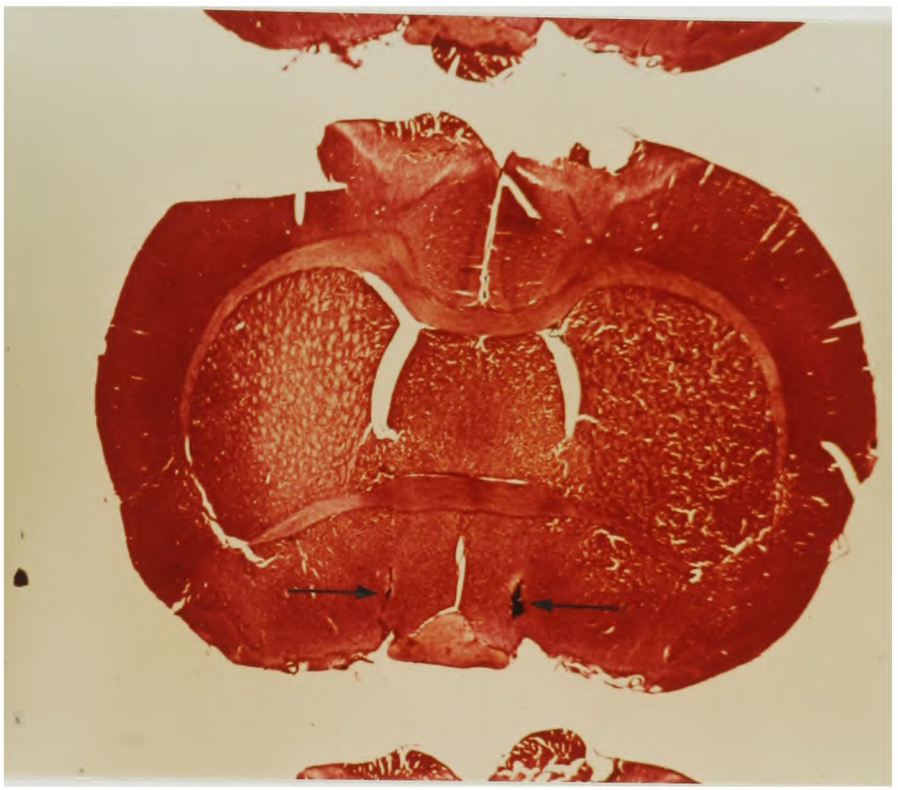


Figure 2.9

Plate. Photomicrograph of neutral red stained coronal section of rat brain. Prussian blue spots (↑) can be seen in the region of the steel electrode terminations (a.c. stimulation).

electrodes achieving with steel electrodes in pentobarbital blocked pro-oestrous rats, full ovulation in 5 of 6 rats, and partial ovulation in the other. The level of d.c. used in this study was much smaller than that applied by Nooter and Zeilmaker (1970) who applied 1.5 mA for 10 sec. in a similar study, and slightly larger than that applied by Quinn (1966) who applied 10 μA for 20, 40 or 60 seconds.

Direct current stimulation of the hypothalamus with a variety of electrode material has been a popular stimulation procedure, used by Moll and Zeilmaker (1966); Terasawa and Sawyer (1969); Kaasjager (1969); Clemens, Shaar, Kleber & Tandy (1971); Kalra, Ajika, Krulich, Fawcett, Quijada & McCann (1971); Gallo, Johnson, Goldman, Whitmoyer & Sawyer (1971); Clemens, Shaar & Smalstig (1972); and Meijs-Roelofs (1972).

Present Experiments

A current of 15 μA was applied to each side of the preoptic area for 10 sec. by means of twin unipolar electrodes. In each case the indifferent electrode was placed in the rectum, the preoptic electrode was the anode, and the stimulus to the right side of the preoptic area followed that to the left side after a pause of 5 sec. The results of these experiments can be seen in Table 2.5 and Figure 2.10. Direct current stimulation with platinum electrodes

Table 2.5

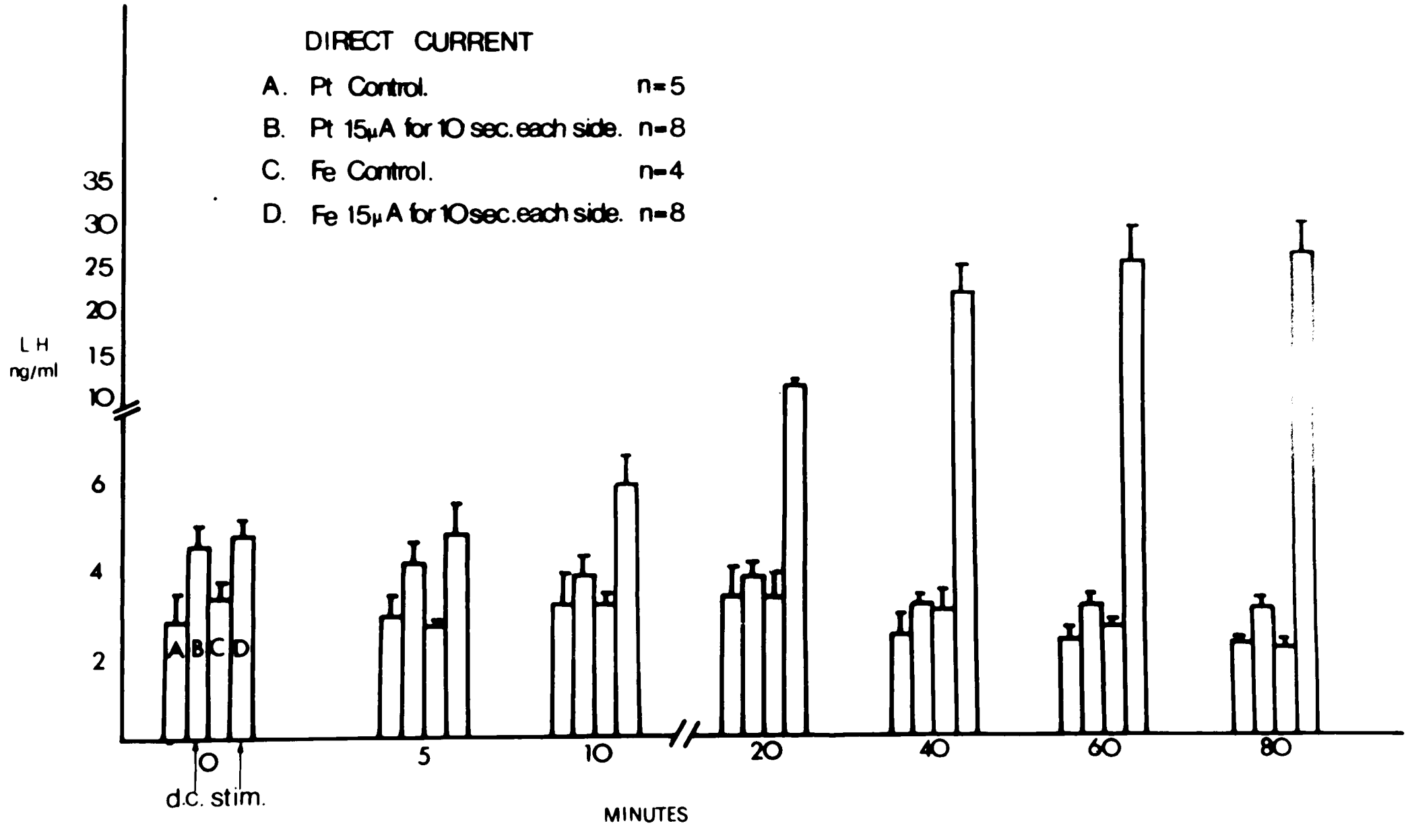
Mean peripheral LH concentrations (± S.E.M.) at various times during and after medial preoptic area direct current stimulation of amplitude 15 μA, duration 10 sec. via steel and via platinum electrodes.

Time (min.)	Pt Control	Pt Stimulation	Fe Stimulation	Fe Control
**Q (mC)	0	0.3	0.3	0
n	5	8	8	4
0	2.8 ± 0.5	4.4 ± 0.5	4.6 ± 0.5	3.2 ± 0.4
5	2.9 ± 0.5	4.0 ± 0.5	4.7 ± 0.7	2.6 ± 0.1
			*p< 0.05	
10	3.1 ± 0.8	3.7 ± 0.5	5.8 ± 0.7	3.0 ± 0.3
			*p< 0.05	
20	3.2 ± 0.7	3.7 ± 0.3	10.6 ± 1.4	3.2 ± 0.6
		*p< 0.001	*p< 0.01	
40	2.3 ± 0.5	3.0 ± 0.3	21.7 ± 3.9	2.9 ± 0.6
		*p< 0.001	*p< 0.01	
60	2.2 ± 0.3	3.0 ± 0.3	25.4 ± 4.0	2.5 ± 0.2
		*p< 0.001	*p< 0.01	
80	2.1 ± 0.1	3.0 ± 0.2	26.4 ± 3.6	2.0 ± 0.2
		*p< 0.001	*p< 0.001	

* Students t test.

** Total coulombage applied.

Figure 2.10 Histogram showing peripheral plasma LH concentrations prior to, during, and after medial preoptic area d.c. stimulation with either platinum or steel electrodes.



with the parameters used here has no significant effect on peripheral LH concentration. By contrast, the same stimulus applied through steel electrodes has a variable but marked effect on peripheral LH concentration, LH concentration being significantly greater than that of control groups from 5 mins. onwards. At the 80 min. sampling period there is no evidence of decline in peripheral LH levels.

Sections of brains stimulated with d.c. via steel electrodes show dense Prussian Blue spots denoting the deposition of iron in the tissues (Figure 2.11). A section of brain stimulated via platinum electrodes appears in Figure 2.12.

2.11. Stimulation Parameters - Anaesthetics, Cerebral Stimulation

Anaesthetics

Cross and Dyer (1970) have used hypothalamic islands ~~to~~ in the investigation of ~~monitor~~ activity of hypothalamic neurones. They reported that while urethane had a negligible effect on neuronal firing rates, methohexitone, a short acting barbiturate has a markedly depressive effect on neuronal activity. Fink (1967a) and Blake and Sawyer (1972) report that urethane in intact female rats blocks ovulation at pro-oestrus when administered in anaesthetic doses. It was decided to examine this effect by stimulating a group of animals in the preoptic area under pentobarbitone sodium anaesthesia (Nembutal, Abbott Labs.;

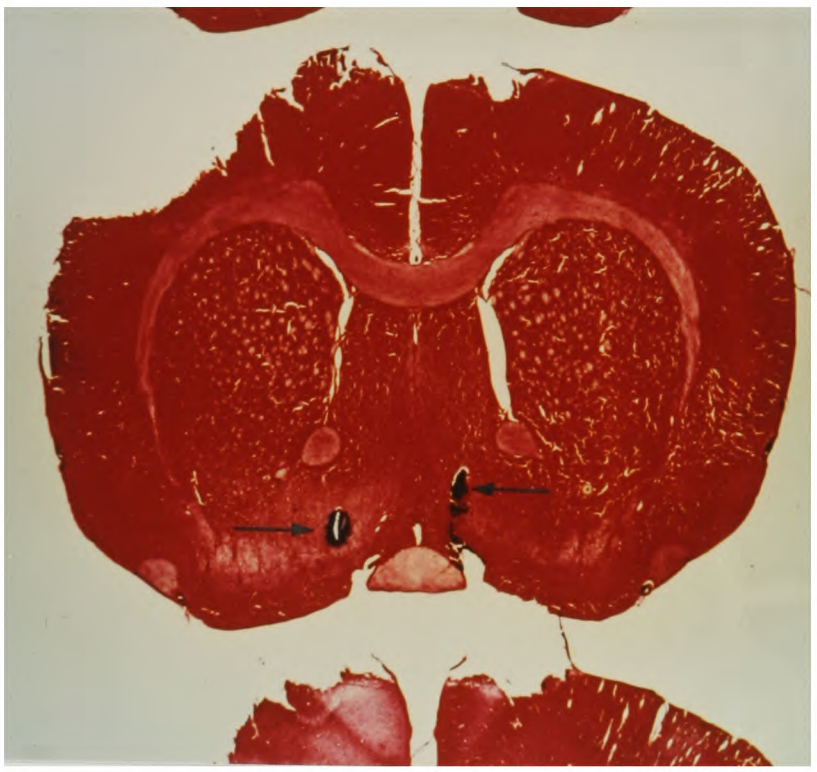


Figure 2.11

Plate. Photomicrograph of neutral red stained coronal section of rat brain. Prussian blue spots (↑) can be seen in the region of the steel electrode terminations (d.c. stimulation).

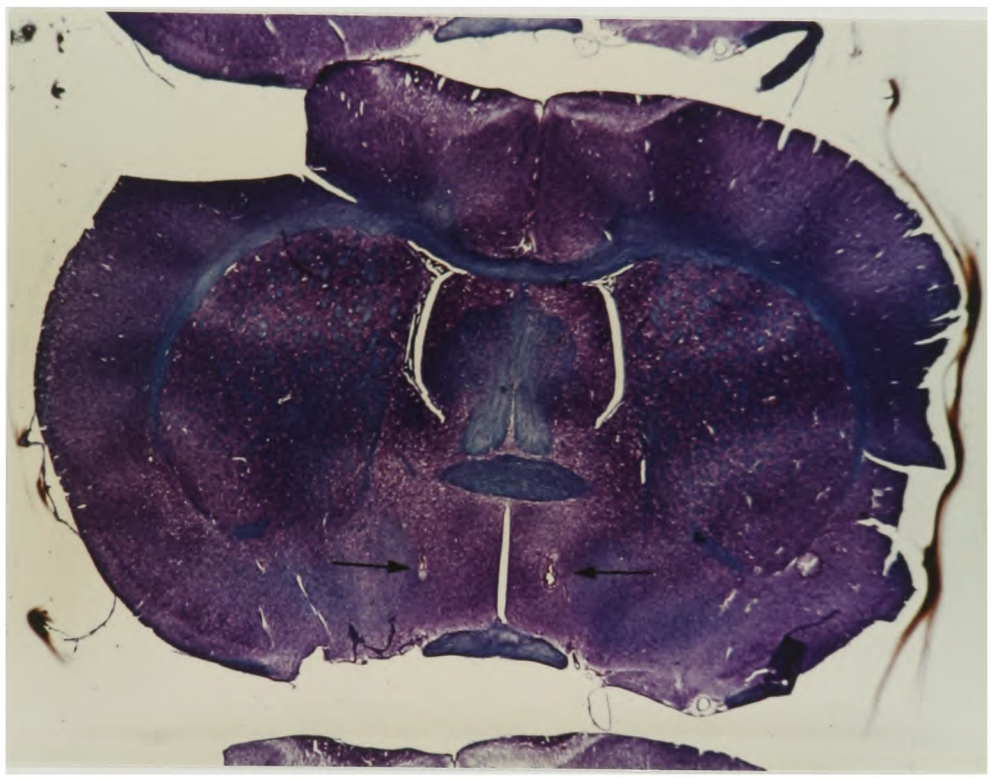


Figure 2.12

Plate. Photomicrograph of thionin stained coronal section of rat brain. Note the electrode terminations (↑) and the area of tissue damage (platinum electrodes, d.c. stimulation).

Kent) at a dose of 36 mg/kg. The stimulus, applied for a ten minute period, 30 sec. on/off, was biphasic pulses, 60 Hz, pulse amplitude 0.5 mA, and pulse duration 1.0 m sec.

The results of this experiment can be seen in Table 2.6 and Figure 2.13. While it would appear that the barbiturate anaesthesia has depressed the response in peripheral LH concentration, at no time is the difference between LH levels achieved under urethane and under Nembutal significant.

Cerebrum Stimulation

It is important in electrical stimulation experiments to ascertain that the observed effects of stimulating a particular area of the brain cannot be achieved by stimulating a remote area of the brain not thought to have functional importance with respect to the area under consideration. If this did prove to be the case, then the observed response might simply be due to wholesale central nervous system "arousal" for example. In the present experiment the cerebrum was electrically stimulated with the same waveform and parameters as in the previous subsection.

The site chosen for stimulation was at the same anteroposterior co-ordinate but 4.1 mm higher in the dorso-ventral plane. This put the electrode tips in the region of the corpus callosum.

The results of such stimulation can be seen in Table 2.6 and Figure 2.13. There is no significant change in peripheral LH

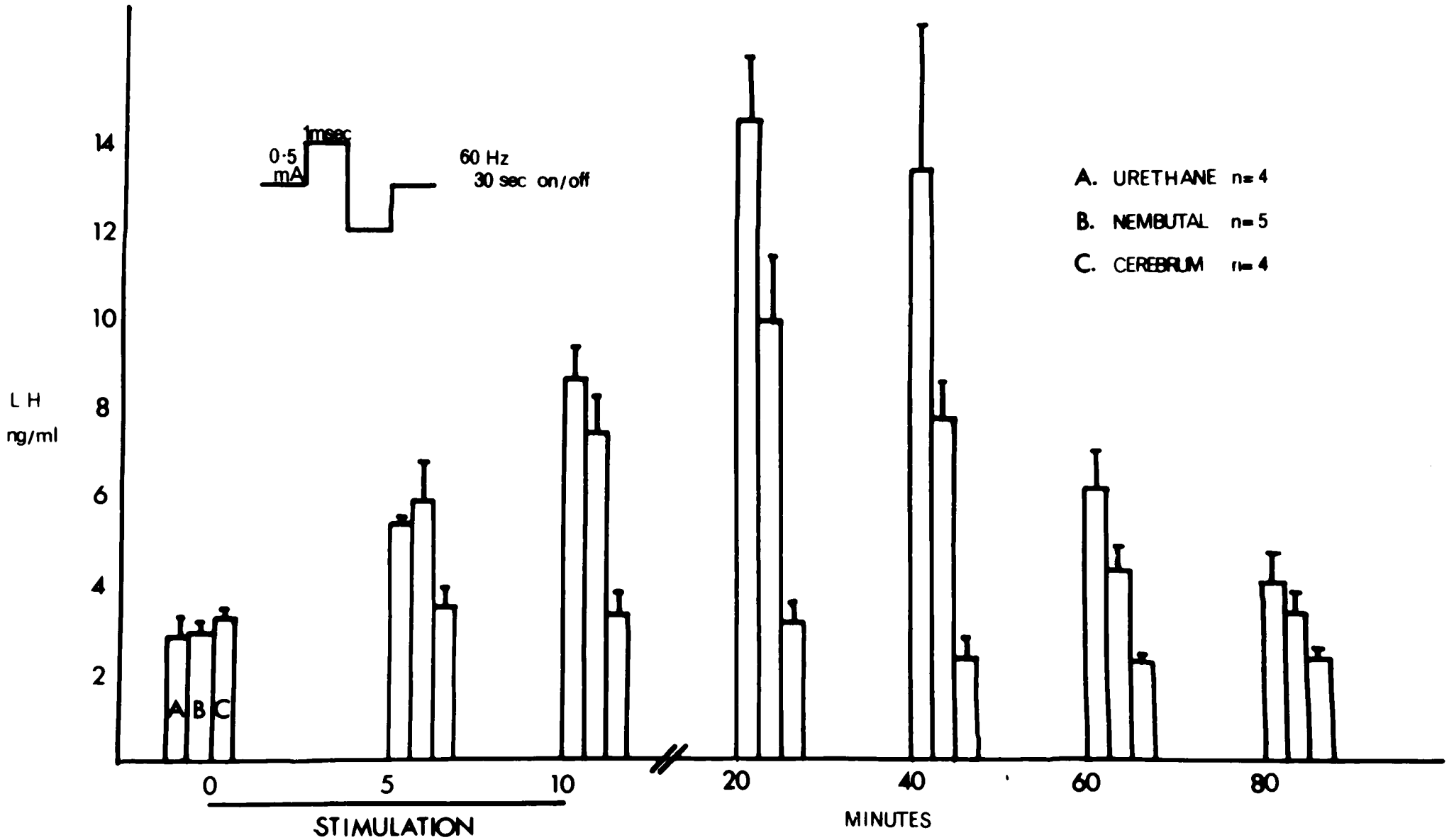
Table 2.6

Mean peripheral LH concentrations (± S.E.M.) at various times during and after biphasic stimulation of the medial preoptic area under Urethane and Nembutal anaesthesia, and the cerebrum under Urethane anaesthesia. Platinum electrodes, 60 Hz, pulse duration 1 msec., amplitude 0.5 mA, 10 min. stimulation 30 sec. on/off.

Time (min.)	Urethane 1g/kg	Nembutal 36 mg/kg	Cerebrum stimulation
Q (mC)	18.0	18.0	18.0
n	4	5	4
0	2.8 ± 0.4	2.8 ± 0.2	3.1 ± 0.2
5	5.4 ± 0.1	5.8 ± 0.9	3.4 ± 0.6
10	8.6 ± 0.7	7.4 ± 0.8	3.3 ± 0.5
20	14.5 ± 1.5	9.9 ± 1.5	3.0 ± 0.5
	*p> 0.05		
40	13.4 ± 3.4	7.7 ± 0.9	2.2 ± 0.6
	*p> 0.1		
60	6.1 ± 0.8	4.2 ± 0.7	2.2 ± 0.2
80	4.0 ± 0.7	3.3 ± 0.5	2.3 ± 0.2

* Students t test.

Figure 2.13 Histogram showing peripheral plasma LH concentrations prior to, during, and after; medial preoptic area a.c. stimulation under urethane or Nembutal anaesthesia, and cerebral a.c. stimulation (platinum electrodes).



concentration as a result of cerebral stimulation over the time period of sampling.

2.12. Stimulation Parameters - FSH Responses

In their studies on pro-oestrous rats blocked with Nembutal, Kalra, Ajika, Krulich, Fawcett, Quijada and McCann (1971) observed that direct current stimulation (100 μA for 60 sec. via steel bipolar concentric electrodes) of the medial preoptic area, the anterior hypothalamic area (AHA) and the median eminence-arcuate nucleus area (ME-ARC) led to rises in peripheral plasma concentrations of LH within one hour. By contrast, elevations in peripheral plasma FSH concentration were obtained only with stimulation of the anterior hypothalamic and median eminence-arcuate nucleus areas and not with stimulation of the medial preoptic area.

Present Experiments

Blood samples of 0.5 ml taken in the experiments described in Sections 2.6 to 2.11 allowed the assay of FSH in each case in single 100 μl samples only while LH was assayed in duplicate 50 μl samples. The peripheral FSH concentrations in these experiments can be found in Appendix 2.3.

The most notable feature was the degree of variability, which was far greater than that for LH. The clear patterns of response to

various experimental treatments observed in plasma LH concentrations are not seen with FSH. Only in the case of d.c. stimulation with steel electrodes (Appendix 2.3, Figure A.5) is there a degree of resemblance to LH responses, and even then the FSH response is not as marked as the LH response.

The responses to biphasic electrical stimulation with platinum electrodes (60 Hz, pulse duration 1 msec., pulse amplitude 0.5 mA, 30 sec. on/off for 10 min.) in the medial preoptic area for both LH and FSH are listed in Table 2.7 and appear in Figure 2.14. In contrast to the significant increases in LH concentration from 5 to 60 min., the rise in FSH concentration is not significant at any time, though it should be stressed again that FSH estimations were made on single samples, while LH estimations were made on duplicate samples.

For purposes of comparison, stimulation with the same parameters was applied in the anterior hypothalamic area of male rats. (Co-ordinates according to de Groot (1959) Anterior 6.8 mm, Down 2.0 mm). In these experiments two 100 µl samples of plasma were taken for FSH assay and one 50 µl sample for LH assay. Results of these experiments can be found in Table 2.8 and appear in Figure 2.15. In this case rises in LH concentration are significant at 10, 20, 40 and 60 min., while rises in FSH concentration are significant at 20 min. Generally, the response profiles in Figures

Table 2.7

Mean peripheral FSH and LH concentrations (± S.E.M.) at various times during and after medial preoptic area stimulation with biphasic pulses at 60 Hz, pulse duration 1 msec., pulse amplitude 0.5 mA, platinum electrodes. 10 min. stimulation, 30 sec. on/off. Q (mC) = 18.0

Time (min.)	0	5	10	20	40	60	80
FSH ng/ml (n = 4)	520 ± 20	570 ± 50	550 ± 40	590 ± 40	660 ± 70	670 ± 80	600 ± 70
P*	-	NS	NS	NS	NS	NS	NS
LH ng/ml (n = 4)	2.78 ± 0.40	5.35 ± 0.14	8.62 ± 0.70	14.50 ± 1.48	13.41 ± 3.38	6.08 ± 0.84	4.00 ± 0.65
P*	-	<0.001	<0.001	<0.001	<0.05	<0.02	NS

*Compared to time 0 (Students t test).

Figure 2.14 Histogram showing peripheral plasma LH and FSH concentrations prior to, during, and after medial preoptic area a.c. stimulation (platinum electrodes).

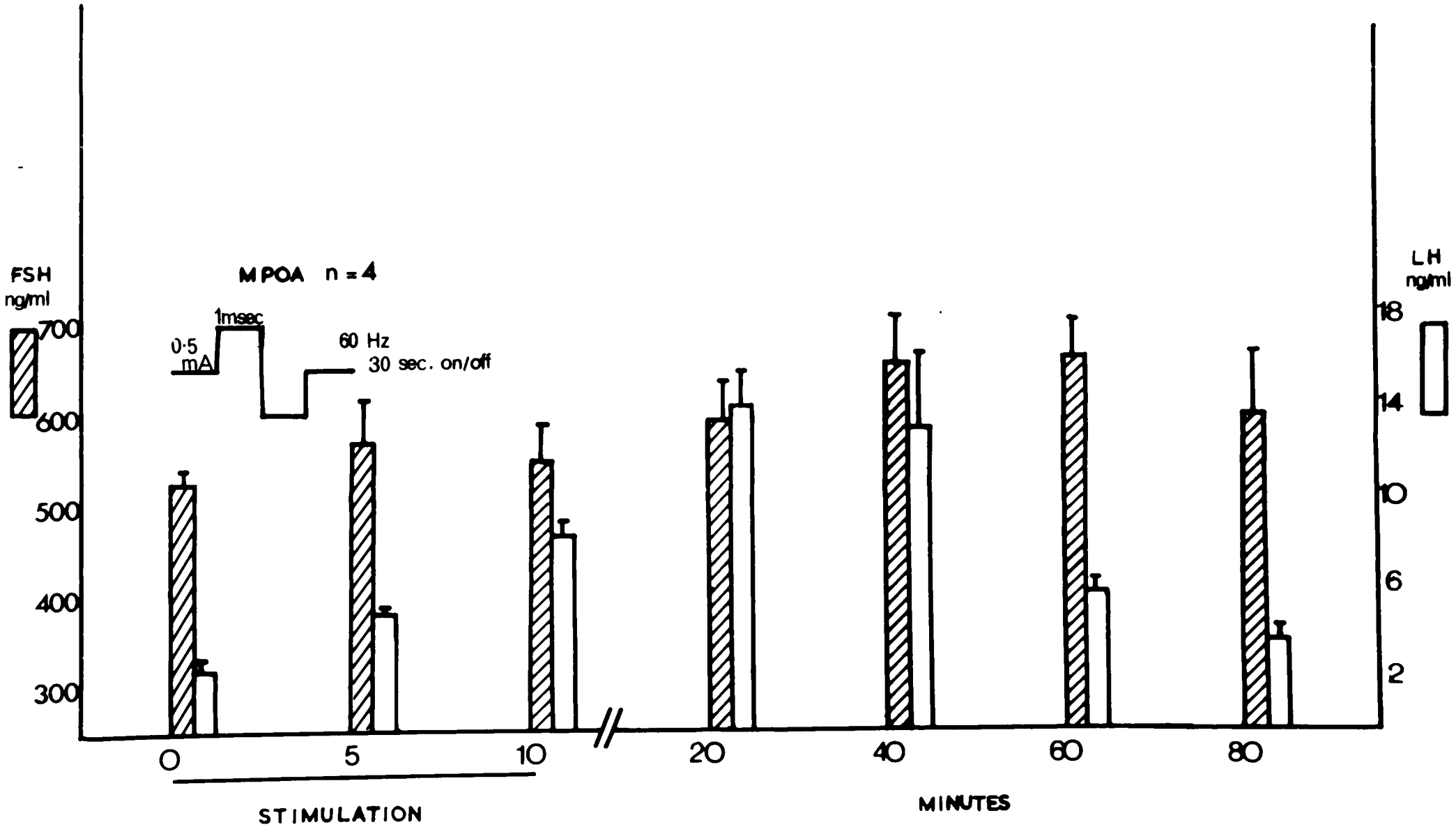


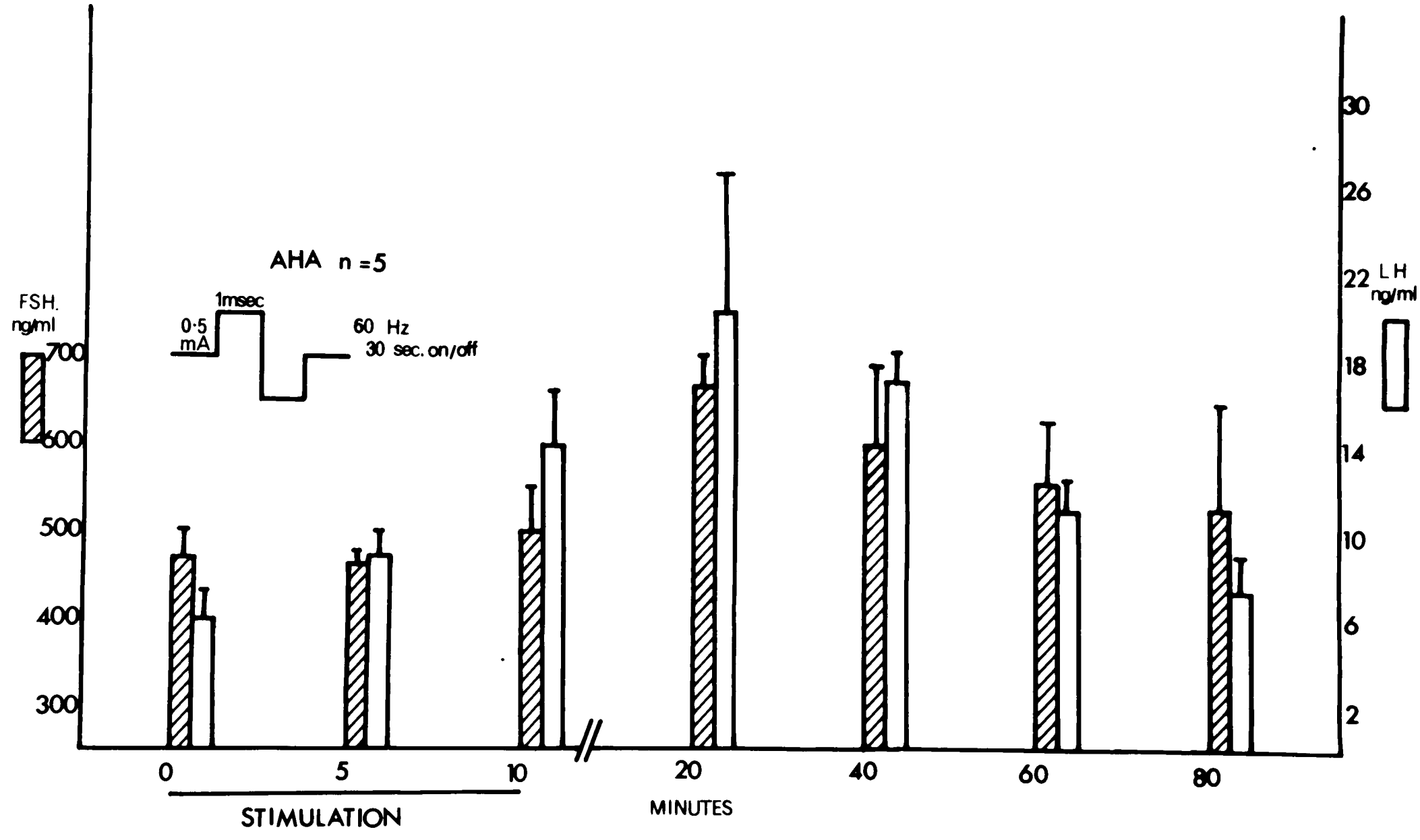
Table 2.8

Mean peripheral FSH and LH concentrations (± S.E.M.) at various times during and after anterior hypothalamic area stimulation with biphasic pulses at 60 Hz pulse duration 1 msec., pulse amplitude 0.5 mA, platinum electrodes. 10 min. stimulation, 30 sec. on/off. Q (mC) = 18.0.

Time (min.)	0	5	10	20	40	60	80
FSH ng/ml (n = 5)	470 ± 28	462 ± 17	502 ± 52	658 ± 45	600 ± 87	556 ± 71	528 ± 121
P*	-	NS	NS	<0.01	NS	NS	NS
LH ng/ml (n = 5)	5.9 ± 1.2	8.8 ± 1.2	13.9 ± 2.6	21.5 ± 5.1	16.9 ± 1.3	11.0 ± 2.7	7.3 ± 1.6
P*	-	NS	<0.05	<0.02	<0.05	NS	NS

*Compared to time 0 (Students t test).

Figure 2.15 Histogram showing peripheral plasma LH and FSH concentrations prior to, during, and after anterior hypothalamic area a.c. stimulation (platinum electrodes).



2.14 and 2.15 for both LH and FSH are similar.

2.13 Stimulation Parameters - Spread

Attempts have been made to study the spread of electrical stimulation in the hypothalamus. The pathway(s) in the hypothalamus controlling LH secretion by the pituitary gland appears to be diffuse and the response to stimulation of such pathways in terms of gonadotrophin secretion cannot be recorded and assessed immediately. However, a clearly defined hypothalamic nerve fibre tract, lacking synaptic relays and controlling a rapid response capable of repetition is the paraventriculo-supraoptico-hypophysial tract through which are regulated the secretion of oxytocin and vasopressin from the posterior pituitary gland. The discharge of oxytocin can be readily monitored by observation of the milk-ejection response (Cross and Harris, 1952). While recent studies of milk-ejection activity have been conducted in rabbits (Harris, Manabe & Ruf, 1969; Urban, Moss and Cross, 1971) and guinea pigs (Tindal and Yokoyama, 1962), it has been demonstrated by Bisset, Clark, Haldor, Harris, Lewis and Rocha e Silva (1967) that the lactating rat is a highly sensitive preparation for such studies.

The aim of the present experiments was to insert an electrode into the paraventriculo-hypophysial tract within the ventral hypothalamus at the level of the infundibular stem, achieve by electrical stimulation a milk-ejection response measured by recording

intramammary pressure, and then to move the electrode a measured distance from its original site and try to repeat the milk-ejection response. If the response was unobtainable, then that particular set of stimulus parameters were such that the stimulus did not spread over that particular distance in that part of the hypothalamus.

Materials and Methods

A single female Wistar rat weighing 300 g. was anaesthetised with an intraperitoneal injection of urethane (10 % solution, 10 ml/kg) on the third day post partum and placed in a stereotaxic machine. An intravenous line was inserted into the left femoral vein (Portex; PP 25) and a cannula was inserted into the primary duct of the lower right inguinal mammary gland and tied firmly into place. The cannula was connected to a strain gauge (Model P 23 AC, Statham; Puerto Rico), the whole system being filled with a 4 % solution of sodium citrate to prevent clotting of milk. The strain gauge was connected to a low-level d.c. preamplifier and pen recorder (Model 50, Grass; Mass.) so as to maintain slight tension on the teat.

Intravenous injections were given in a volume of 0.05 - 0.15 ml washed in with 0.1 ml of 0.9 % saline solution.

A stainless steel unipolar electrode was introduced into the region of the infundibular stem in the midline (co-ordinates according to de Groot (1959), anterior 6.2 mm, down 3.1 mm). An

indifferent electrode was inserted into the rectum. Stimuli consisted of single 30 sec. trains of biphasic impulses, duration 1 msec., amplitude 0.05 -1.0 mA at a frequency of 50 Hz. A period of 5 min. rest was allowed after each positive milk-ejection response. At the end of the experiment d.c. (25 μA, 10 sec.) was passed through the intracranial electrode (as anode), at several sites and 40 μm frozen sections were treated with 2 % potassium ferrocyanide solution to develop Prussion Blue reactions, and stained with haematoxylin and eosin.

Milk ejection response waves were traced onto cartridge paper, inked in and their areas were measured with an image analysing computer (Model 720, Quantimet; Cambridgeshire).

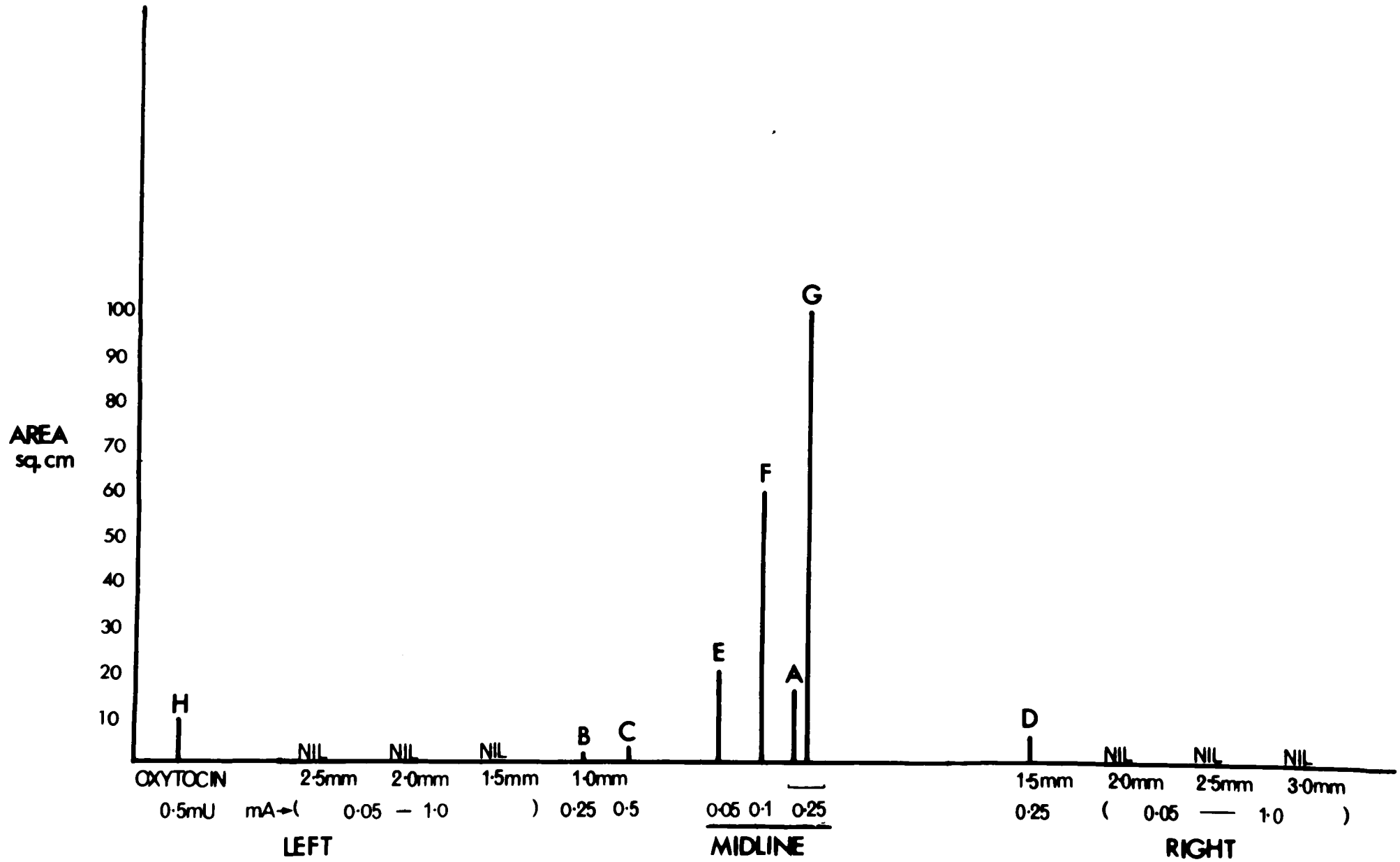
Oxytocin injected was synthetic oxytocin (Syntocinon, Sandoz; London) diluted in normal saline.

Results

The results are presented in the histogram (Figure 2.16), where the horizontal ~~x-axis~~ represents the stimulation sites tested, and the vertical ~~y-axis~~ the area under the curves of the corresponding milk ejection responses.

Initially response A was obtained in the midline with pulse amplitude 0.25 mA. The electrode was then shifted to a point 2.5 mm to the right of the midline at the same anteroposterior and vertical

Figure 2.16 Histogram showing milk ejection responses to a.c. (steel electrode) stimulation at various pulse amplitudes, and at various sites in relation to the paraventriculo-hypophysial tract (see text, Section 2.13).



co-ordinates. Stimulation at this site, and at 2.0 mm and 1.5 mm right of the midline gave no milk ejection response despite increases in pulse amplitude to 1.0 mA. At 1.0 mm to the right small responses were obtained with pulse amplitudes 0.25 mA (response B) and 0.5 mA (response C).

The electrode was then shifted to a point 3.0 mm to the left of the midline. Testing here and at 2.5 mm and 2.0 mm from the midline did not elicit milk ejection responses. However, at 1.5 mm left, response D was obtained with a pulse amplitude of 0.25 mA. The electrode was then returned to the original midline position where stimuli with pulse amplitudes of 0.05 mA (E) 0.1 mA (F) and 0.25 mA (G) produced large responses, the difference between the original response at 0.25 mA (A) and the second response in the midline at 0.25 mA (G) being marked.

Finally an intravenous injection of 0.5 mU oxytocin produced response H.

Direct current was passed at three points, 1.5 and 2.5 mm right and at 2.5 mm left, and the resulting evidence of deposition of iron can be seen in Figure 2.17.



Figure 2.17

Plate. Photomicrograph of H + E stained coronal section of rat brain. Prussian blue spots (↑) can be seen at sites of stimulation in milk-ejection experiment (see text, Section 2.13).

2.14. DISCUSSION

Although many studies have been carried out on the activation of LH release in female rats by electrical stimulation of the hypothalamus (either by measurement of peripheral LH, or indirectly by observation of ovulation), there have been very few reports of this phenomenon in males. Moll and Zeilmaker (1966) applied d.c. at the high amperage of 1.5 mA for 10 sec. via unipolar steel electrodes to young castrated male rats bearing ovarian transplants. After such stimulation in the median eminence and medial pre-optic area, they found histological changes in the ovarian grafts suggestive of the discharge of gonadotrophins, namely displacement of ova towards the centre of follicles, "loosening" of cumulus oophorus, and corpus luteum formation, in follicles containing entrapped ova. Sham stimulated animals showed only follicles in various stages of development with some ripe follicles but no corpora lutea. While "ovulation" in the normal sense did not occur, this failure of ova to leave follicles is usual in transplanted ovaries following ovulation-inducing doses of gonadotrophin (Noyes, Yamate & Clewe, 1958).

Moll and Zeilmaker's findings were confirmed in a contemporaneous report by Quinn (1966) who stimulated young male rats at age 30 days bearing ovarian transplants. Stimulation in the medial preoptic area with concentric bipolar steel electrodes at the

much lower amperage of 10 μA for 60 sec. produced luteinisation in eight of nine rats. In addition, injection of luteinising hormone (NIH-LH-B1, 8 μg/100 g body weight) at a known ovulating dose, produced in this study marked luteinisation in both of two animals tested.

The present study provides direct evidence for the release of luteinising hormone from the anterior pituitary gland consequent upon electrical stimulation of the medial preoptic area of the diencephalon of the normal male rat, and confirms the preliminary studies of Burger, Fink and Lee (1972) who achieved rises greater than 400 % in peripheral LH concentration in three male rats after stimulation of the medial preoptic area. In the female rat, the centre for the neural mechanism that triggers the burst of LH secretion responsible for ovulation is probably located in the preoptic area (Bunn and Everett, 1957; Critchlow, 1958; Flerko, 1970). While there is no equivalent defined cyclical waxing and waning of gonadotrophin release in the male rat the preoptic area may be involved in the post-coital release of pituitary LH observed by Taleisnik, Caligaris & Astrada (1966).

In addition, Saginor and Horton (1968) have observed a rise in plasma testosterone and androstenedione in male rabbits during coitus. These last two observations need confirmation as Dr.K.Brown-Grant &

Ms.F. Murray (personal communication) have been unable to find any rise in plasma testosterone after coitus in male rabbits. It is also pertinent to mention that the copulatory behaviour of the male is significantly affected by lesions of the preoptic area (Lisk, 1968; Giantonio, Lund & Gerall, 1970) and that the restorative effect of testosterone on the consummatory behaviour of castrated rats is particularly marked when this steroid is implanted in this region of the brain (Davidson, 1966a).

Moll and Zeilmaker (1966) found, in addition to their findings with preoptic stimulation, that electrochemical stimulation in the region of the median eminence was also capable of producing "ovulation" in transplanted ovaries. Indeed, they were unable to distinguish histologically between ovaries after median eminence stimulation, and ovaries after preoptic stimulation. While the present study did not include median eminence stimulation, other evidence confirms that the median eminence is of central importance in the control of pituitary gonadotrophin secretion in the male, as in the female. There are several sources of evidence for this conclusion. Lesions in the median eminence region of rats (and cats, rabbits and sheep) result in testicular atrophy (Davidson, 1966b). While these lesions can disrupt the hypophysial system, the resulting inhibition of gonadotrophin secretion cannot be explained by pituitary infarction (Davidson, 1966b). In addition crude and

purified extracts of the median eminence and basal hypothalamus release LH (Johnson, 1964; Schally, Carter, Arimura & Bowers, 1967), and FSH (David, Fraschini & Martini, 1965; Saito, Arimura, Muller, Bowers and Schally, 1967), when administered to male rats.

It might be argued that in the present studies the discharge of gonadotrophin might be solely from the median eminence, and that the stimulus effect had "spread" from the preoptic area to the median eminence producing gonadotrophin discharge directly from the median eminence, rather than indirectly via the preoptic area neural elements. The report by Everett (1965), that while biphasic stimulation with platinum electrodes in the medial preoptic area produced full ovulation in female rats (blocked with pentobarbital on the afternoon of pro-oestrus), the same stimulation in the lateral preoptic area produced largely negative results, argues against this possibility. In addition, in the present study on milk ejection responses, there is evidence (subject to several caveats, see below) that the optimal biphasic stimulus parameters used are such that "spread" of the stimulatory effect does not occur over distances more than 1.0 - 1.5 mm in the ventral hypothalamus. According to de Groot (1959) the distance separating the midpoint of the arcuate nucleus-median eminence area from the midpoint of the pre-optic area is of the order of 1.8 - 2.2 mm.

Comparison of effects of medial preoptic area stimulation on peripheral LH concentration between male and female animals demonstrates that there are marked differences in responsiveness depending on the time in the oestrous cycle in the female at which stimulation occurs. Under Nembutal anaesthesia, the increment in LH concentration in male animals at 20 min. is 7.1 ng/ml (Table 2.6), while at 13.30 hours of dioestrus the increment in female rats stimulated with the same parameters is 6.3 ng/ml, the initial peripheral LH concentrations being 2.8 and 1.9 ng/ml respectively. By contrast, at 18.00 hours of pro-oestrus the increment obtained at 20 min. after stimulation is 120.7 ng/ml, the initial peripheral LH concentration being 20.2 ng/ml (Fink & Aiyer, 1974).

This marked difference in response to stimulation in female rats at pro-oestrus and dioestrus is probably related to the increase in sensitivity of the anterior pituitary gland which has been also shown to occur at pro-oestrus in response to intra-venous injections of synthetic luteinising-hormone releasing factor (Aiyer, Fink & Greig, 1974). These findings, however, do not necessarily mean that the female preoptic area-median eminence axis reactivity to electrical stimulation at pro-oestrus is not greater than that of male animals.

In their studies on the effects of electrochemical stimulation in the hypothalamus, Kalra et al. (1971) demonstrated that FSH release was produced only when the stimulating electrodes were situated in the

AHA and ME-ARC area, and not when electrodes were situated in the preoptic area or septal complex of female rats on the afternoon of pro-oestrus. The results presented in Section 2.12 cannot be considered as being confirmatory of this view in male animals although a significant rise in peripheral FSH concentration was achieved at 20 min. with biphasic stimulation in the AHA, while no significant rise was found after stimulation in the medial preoptic area. Detailed comparisons between the results are rendered inappropriate because of measurements made on single samples of peripheral plasma for LH in the AHA and for FSH in the medial preoptic area. There is again questionable evidence that electro-chemical stimulation in the medial preoptic area can cause realease of FSH (Appendix 2.3, Figure A-5) which observation would be in conflict with the results of Kalra et. al. (1971) for female rats. In conclusion, the present data are too limited to draw any firm conclusions about any spatial differences between areas of the male hypothalamus involved in LH release, and those involved in FSH release, if indeed such differences exist.

Other studies of parameters of stimulation with electric currents in the hypothalamic area include that of Harris, Manabe & Ruf (1969), using the supraoptico-hypophysial tract and the milk-ejection reflex in the rabbit, and that of Martin, Kontor & Mead (1973), using the ventromedial hypothalamic nucleus and plasma

growth hormone concentration as, in each case, stimulus site and peripheral response respectively. Harris and his colleagues using unipolar and bipolar platinum electrodes, compared the effectiveness of sine wave and square wave a.c. stimulation at 50 Hz and pulse amplitude 0.25 - 0.75 mA. When the pulse duration of the square wave was 3 msec. (the pulse duration of the sine wave being 10 msec.) the two types of stimulation were equally effective. The important point is that the charge passed in each pulse of the square wave pulse (1.5 μC) is less than half that of each pulse of the sine wave at that frequency (3.17 μC). As a consequence, lesioning of the area being stimulated is less likely with square wave pulses. Upon examination of square wave stimulation further, Harris and his colleagues concluded that balanced biphasic pulses at 50 Hz with a pulse duration of 3 msec. and pulse amplitude of 0.02 - 0.50 mA were recommended for stimulatory investigation of control of the posterior pituitary by the hypothalamus. In later work on the female rat (Harris and Ruf, 1970) biphasic pulses at 30 Hz, with a pulse duration of 1 msec., and pulse amplitude 0.5 mA were used in stimulation of the medial preoptic area.

Martin and his colleagues (1973) stimulated male rats in the ventromedial nucleus of the hypothalamus and measured resulting changes in plasma growth hormone concentration. They found that with unipolar nichrome electrodes implanted bilaterally and biphasic

square waves, stimulation at 60 Hz was more effective than at 15 Hz. A pulse duration of 2 msec. was more effective than one of 0.2 msec. and pulse amplitude of 0.5 - 0.7 mA was more effective than one of 0.1 - 0.3 mA.

In broad terms, the present studies confirm that for hypothalamic stimulation employing biphasic square wave stimulation, optimum parameters of stimulation are frequency 60 Hz, pulse duration 0.5 - 1.0 msec. and pulse amplitude 0.5 mA. Clearly the constraints of time and expense do not allow of trying every permutation of the three parameters mentioned. Nevertheless, in this particular case, it is important to appreciate the fact that beyond certain limits, increasing the total amount of charge applied via the electrodes does not result in an increase in the response measured. The charge passed per pulse pair is 0.5 - 1.0 μC, and the total charge in each 30 sec. pulse train is 1.8 - 3.6 mC. These are tiny amounts of charge. The elegant study of Rowland, MacIntyre and Bidder (1960) using a variety of electrode materials for biphasic stimulation of cat cerebral cortex showed that at the light microscope level, provided frequency of stimulation is 20 Hz or greater, no observable injury (other than the electrode track) is seen when the coulombage per pulse pair is less than 20 μC, and when no more than 10 C are passed in any one train of pulses. This suggests that the safety factor in the present experiments is enormous.

The difference between the effects of biphasic stimulation passed via platinum and steel electrodes is of importance. It is clear that stimulation with steel electrodes has a more pronounced and longer lasting effect on plasma luteinising hormone concentration elevation. To achieve equivalence of effect when using platinum electrodes it might be necessary to stimulate for longer periods. In their studies on stimulation of the ventral hippocampus, Gallo, Johnson, Goldman, Whitmoyer and Sawyer (1971) failed to obtain with platinum electrodes an enhancement of hypothalamic electrical activity that was evident with steel electrodes.

This point becomes more ~~cogent~~ important when d.c. stimulation is considered. The failure of d.c. stimulation via platinum electrodes to raise plasma LH levels, while stimulation through steel electrodes had a marked and continued effect over the time period studied, has been observed before. Everett and Radford (1961) found that female rats failed to ovulate when stimulated with 500 μA d.c. for 30 seconds through platinum electrodes, whereas ovulation was consistently obtained with steel electrodes at 100 μA for 30 sec. They completed their study with the observation that the injection of 1 μl of 5 % $FeCl_3$ into the same medial preoptic area was sufficient to cause consistent ovulation. Doubtless both electrode materials have their particular applications, but the fact that responses to stimulation with steel electrodes result from the "irritant effect" of deposits

of ferric ions must be borne in mind. The changes induced by the presence of these deposits are prolonged in comparison to changes produced with platinum electrodes, with which there can be no deposition of metallic ions.

The total charge delivered in d.c. experiments was 0.3 mC (150 μC to each side). By comparison, in biphasic a.c. stimulation with optimal parameters the total charge delivered was 9 - 18 mC. Hence, despite a 30 - 60 fold reduction in coulombage, d.c. stimulation via steel electrodes raised peripheral LH levels at 40 min. to almost twice the levels obtained with a.c. via platinum electrodes. Similarly, but to a lesser extent, d.c. stimulation with steel electrodes was more effective than a.c. stimulation with steel electrodes.

While no measurements of lesion sizes have been made in the present experiments, the differences in lesion size apparent in a comparison of Figure 2.4 (platinum electrodes, a.c.) and Figure 2.12 (platinum electrodes, d.c.) demonstrate that tissue damage in the vicinity of the electrode tip is greater when d.c. stimulation is used. Rowland, MacIntyre & Bidder (1960) compared d.c. with a.c. stimulation passing the same coulombage in each case and found that the size of the lesion produced by a.c. was much smaller than that produced by d.c.

In the present experiments, the intracranial electrode for d.c. stimulation was the anode. This is important, because during the electrolysis of saline solution with steel electrodes, gas is evolved at the cathode. This results in cavitation which increases the size of the lesion produced. Even when twin unipolar electrodes were inserted in the present study, d.c. was passed sequentially between each electrode and an indifferent electrode, so that both intracranial electrodes were anodic.

It is clear from the present studies that the medial preoptic area neurones (no distinction can be made here between nerve cells originating in the preoptic area and fibres of passage whose cell bodies lie for example in the septal complex) which appear to influence the release of LH are responsive both to direct and alternating current stimulation through steel electrodes and to alternating current stimulation through platinum electrodes. They appear to be more sensitive to electrochemical stimulation. The irritative effect of metal ion deposition may continue after stimulation has ceased whereas stimulation with platinum electrodes may activate relatively few fibres, and cease to activate them when the stimulus is halted. Lincoln, Van der Schoot and Zeilmaker (1973) have shown that in pro-oestrous female rats and in adult castrated male rats with ovarian grafts, ovulation occurs after electrochemical stimulation of the medial preoptic area (1 mA,

20 sec.). An EEG change persisted for 90 minutes after application of electrochemical stimulation consisting of conversion from a pattern of alternating sleep and wakefulness, to one of continuous arousal. With platinum electrodes and the same direct current parameters, neither such EEG change nor ovulation was observed. Electrochemical stimulation of the anterior thalamus and amygdala had no effects on either EEG patterns or on ovulation. These authors remark that while it would be inappropriate to assign a causal relationship between ovulation and the changes in EEG following electrochemical stimulation of the hypothalamus, the EEG response involves a change in neuronal activity, as does ovulation. This may suggest that projections of medial preoptic area neurones concerned with control of gonadotrophin release may influence brain areas other than the arcuate nucleus-median eminence area when under electrochemical stimulation.

Whatever the differences between the results of the variety of stimulus procedures used in the present study, great caution is urged when reporting stimulation and lesion experiments. Not only should the electrical parameters including coulombage of pulses and pulse trains be reported, but also the physical and chemical characteristics of the electrodes themselves should be described. In experiments where repeated stimulation procedures are contemplated, the apparent short term effect of platinum electrodes may be advantageous.

The problem of spread of stimulatory effect from the electrode site has been already mentioned briefly. The results of the experiment described in section 2.13 suggest that biphasic pulses delivered for 30 sec. at 50 Hz, with pulse duration 1 msec. and amplitude of up to 1.0 mA are incapable of affecting a sufficiently large mass of nervous tissue in the ventral hypothalamus for a stimulatory effect to "spread" a distance of more than 1.5 mm. Clearly, the evidence for this statement does not exclude a "spread" that, in respect of the milk ejection response, was subthreshold. The results cannot be explained on the basis of exhaustion either of the neural tract or of the mammary gland, since responses E, F, G and H chronologically follow responses A, B and C. The marked difference between responses A and G is difficult to explain unless minute differences in electrode position due to re-introduction of the electrode into the midline can produce a 6-fold increase in response to the same stimulus.

In the assessment of this experiment there are several difficulties to be considered. First, the sheer complexity of hypothalamic pathways means that it is difficult to assess whether oxytocin release after stimulation away from the midline can be attributed specifically to stimulation of the paraventriculo-supraoptico-hypophysial tract, or whether pathways afferent to this tract are responsible. Movement of the electrode tip laterally takes

the electrode tip towards the medial fore-brain bundle in which (at least in the guinea pig) one of the elements of the ascending afferent limb of the milk ejection reflex runs (Tindal & Knaggs, 1971). In addition Urban, Moss and Cross (1971) point out that reproducibility of milk ejection responses from positive stimulation sites in the rabbit brain is extremely poor both between and within experiments, an observation confirmed in the present study. They also comment that "the use of intramammary pressure to monitor release of oxytocin from the neurohypophysis is not free from pitfalls".

Secondly, repetitive stimulation of the milk ejection pathway may lead to fatigue of the nerve fibres concerned, or depletion of stored oxytocin to such an extent that full responses are no longer obtained. Periodic "calibration" of the mammary gland with injected doses of oxytocin may itself lead to fatigue of the mammary gland.

Thirdly, the repeated insertion of electrodes into a small area of tissue may produce sufficient damage and tissue reaction to interfere not only with function, but also with exact histological localisation of electrode sites.

CHAPTER 3

ASSAYS FOR THE LUTEINISING HORMONE-RELEASING FACTOR

3.1. SUMMARY

After consideration of the neurohumoral hypothesis regarding the control of the anterior pituitary gland by the hypothalamus, and of the discovery of the luteinising-hormone releasing factor (LH-RF) in hypothalamic extracts, methods for the bioassay and radioimmunoassay of LH-RF are described. Some applications of bioassay to the measurement of the luteinising hormone releasing activity of synthetic analogues of LH-RF, and of radioimmunoassay to the measurement of LH-RF in a variety of materials are discussed.

3.2. INTRODUCTION

The discovery in extracts of hypothalamic tissue and in extracts of pituitary portal blood of a substance which causes the release from the anterior pituitary gland of gonadotrophins (LH and FSH) has led to the investigation of techniques for the qualitative and quantitative assay of such substances. In the paragraphs that follow, investigations into in-vitro bioassays and a radioimmunoassay for luteinising-hormone releasing factor (LH-RF) are described.

SECTION I BIOASSAYS

3.3. HISTORY

A good deal of evidence indicates that the secretion of pituitary hormones is under the control of the hypothalamus, and there is strong support for the concept first clearly proposed by Green and Harris (1947) that the hypothalamus exerts its effects on the anterior pituitary gland not through purely nervous pathways but through secretion of neurohumoral substances carried to adeno-hypophysial cells by means of the hypophysial portal circulation. Harris (1955) pointed out that following interruption of the portal vessels in the pituitary stalk, changes in the function of those endocrine glands (thyroid, adrenal cortex, testis, ovary) acted upon by anterior pituitary hormones provided good evidence that the maintenance of normal secretory activity of the anterior pituitary gland might depend at least in part on an intact portal blood supply. To Harris (1955) this relationship between anterior pituitary function and the portal blood supply was explicable if hypothalamic nerve fibres were to liberate "hormonal" substances from their axon terminals into capillaries in the median eminence of the hypothalamus; whence these substances were carried to the anterior pituitary gland where they stimulated or inhibited the release and/or synthesis of the various anterior pituitary hormones.

It had been noted however, that ischaemic necrosis of part of the anterior pituitary followed section of the pituitary stalk in the toad (Houssay, 1949), the rat (Harris, 1950b; Barrnett & Greep, 1951; Daniel & Pritchard, 1956) and the sheep and goat (Daniel & Pritchard, 1957, 1958). Donovan and Harris (1954) expressed the view that the amount of anterior pituitary tissue destroyed by stalk section could not account for the impairment of function which occurred since Smith (1932) had demonstrated that as little as 30 % of anterior pituitary tissue was capable of maintaining normal function of the target organs. Harris and Jacobsohn (1952), in a classic investigation, placed pituitary grafts into hypophysectomised adult rats under the median eminence, under the temporal lobe of the brain, and into the hypophysial capsule. The grafts became vascularised in all three sites, but whereas grafts under the median eminence acquired vascular connections with the primary plexus of the portal vessels, those in the other two sites did not. Many animals with grafts under the median eminence exhibited normal target organ function while those with grafts elsewhere showed very little (if any) function of target organs. Similar experiments were carried out by Nikitovitch-Winer and Everett (1958, 1959) and Smith (1961). These observations provided good evidence in support of the neurohumoral hypothesis, for, while it may be appropriate to attribute some of the functional defects which follow simple section of the pituitary stalk to

partial necrosis of the anterior pituitary, such criticism does not apply here. It should be pointed out however, that there is substantial evidence that the pituitary secretes prolactin when its connections with the hypothalamus are severed (Meites, Nicholl and Talwalker, 1963; Everett, 1966).

Other physiological, pharmacological and anatomical data accumulated (Harris, 1955; Markee, Sawyer and Hollinshead, 1946), including identification of pituitary portal vessel systems in other vertebrates (see Green, 1947, 1951) than the laboratory animals in which Harris' original observations had been made, supporting the neurohumoral concept of regulation of the anterior pituitary gland. However, direct evidence for the existence of specific hypothalamic neurohormones involved in release of anterior pituitary hormones was lacking until Saffran and his co-workers (Saffran and Schally, 1955a; Saffran, Schally & Benfey, 1955; Schally, Saffran and Zimmerman, 1958) and Guillemin and his co-worker (Guillemin, 1955a, 1955b; Guillemin and Rosenberg, 1955) provided evidence of the existence of a substance in hypothalamic tissue capable of causing the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. Direct evidence for the occurrence of hypothalamic substance(s) which acted so as to release gonadotrophins soon followed. Harris (1960), and Campbell, Feuer, Garcia & Harris (1961) showed that infusion of median eminence extracts directly

into the pituitary gland was effective in inducing ovulation in rabbits; Nikitovitch-Winer (1962) achieved similar results in rats. McCann, Taleisnik and Friedman (1960) demonstrated the presence of a hypothalamic LH-RF in stalk-median eminence extracts. Clearly the characterisation of these hypophysiotrophic factors depended on the development of reliable, specific, sensitive direct assay methods.

In the classical type of bioassay, an "unknown" preparation is assayed against a "reference" preparation, and several groups of investigators attempted to isolate the substance(s) from the hypothalamus which might provide such a preparation.

After Schally and his co-workers (Schally, Arimura, Bowers, Kastin, Sawano & Redding, 1968; Kastin, Schally, Gual, Midgley, Bowers & Diaz-Infante, 1969; Kastin, Zarate, Midgley, Canales & Schally, 1971) had painstakingly processed and purified hundreds of thousands of porcine hypothalami, they found that a highly purified extract stimulated release of both LH and FSH in men and women, and induced ovulation in a secondary amenorrhoeic (when used in conjunction with human menopausal gonadotrophin). They proceeded to isolate from this porcine material a substance capable of releasing both LH and FSH from the anterior pituitary, and finally determined its structure as a decapeptide (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White, 1971) and synthesised it (Matsuo,

Arimura, Nair & Schally, 1971). Similar work (Amoss, Burgus, Blackwell, Vale, Fellows & Guillemin, 1971) on ovine hypothalami resulted in the extraction of the same decapeptide as that reported by Schally et al., (1971), and synthesis of the decapeptide was also carried out in other laboratories (Monahan, Rivier, Burgus, Amoss, Blackwell, Vale and Guillemin, 1971; Sievertsson, Chang, Bogentoft, Currie, Folkers & Bowers, 1971). These synthetic preparations all proved to have the same LH and FSH releasing activity as purified natural LH-RF (Schally, Arimura, Carter, Redding, Geiger, Konig, Wissman, Jaeger, Sandow, Yanaihara, Yanaihara, Hoshimoto & Sakagami, 1972; Schally, Redding, Matsuo & Arimura, 1972).

The structure of LH-RF appears in Figure 3.1.

In vivo bioassays

Since the discovery of LH-RF, a number of tests have been developed to detect the presence of the factor and more importantly to quantitate its activity. In all bioassays, an estimate must be made of the effect of the factor on LH secretion by the pituitary gland. This means that all bioassays will incorporate in them the variability inherent in the measurement of LH. In in-vivo bioassays, a possible endpoint used for estimation of LH secretion has been ovulation, which suffers from the disadvantage that it is in essence an all or none phenomenon, although the number of ova shed can be

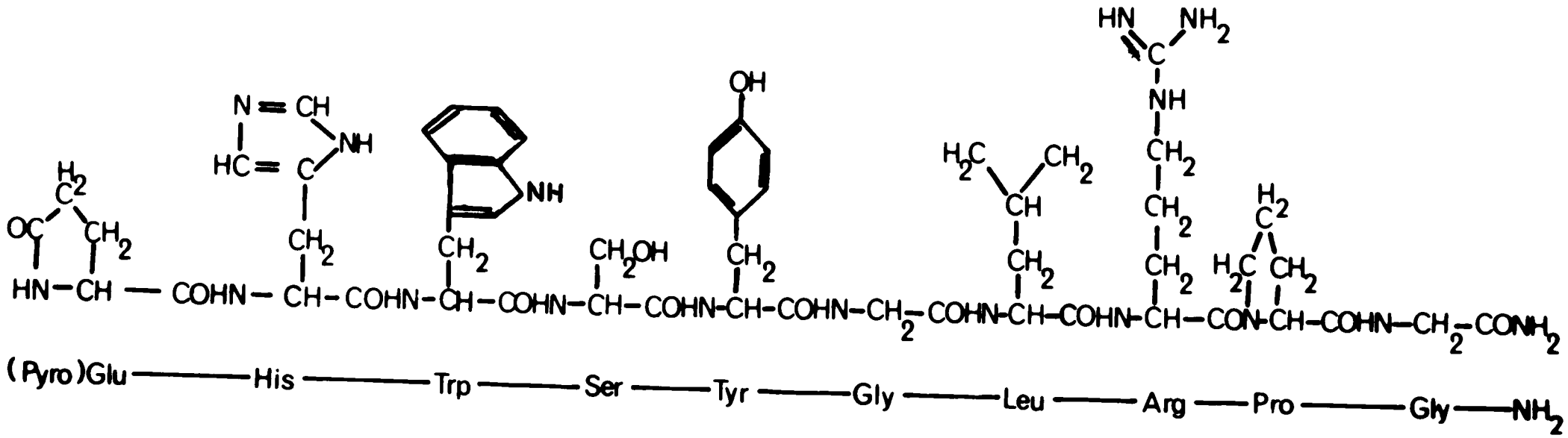


Figure 3.1. Molecular structure of LH-RF.

counted. Furthermore, ovulation can hardly be considered for use as a specific assay of LH secretion, as ovulation in the rat is known to be associated with discharge of both FSH and LH (Caligaris, Astrada & Taleisnik, 1967; McClintoch & Schwartz, 1968).

Another method for the measurement of LH has been the ovarian ascorbic acid depletion (OAAD) assay of Parlow (1958). This assay utilises the fact that LH causes depletion of ovarian ascorbic acid in immature rats made pseudopregnant with injections of pregnant mare's serum and human chorionic gonadotrophin. It is a sensitive assay of LH, Parlow (1961) finding it to be sensitive to as little as 0.075 µg of LH, and specific, although vasopressin, in large doses, will cause OAAD by a direct action on the ovaries (McCann and Taleisnik, 1960). This method was used by McCann, Taleisnik & Friedman (1960) who assayed median eminence extracts with it. The OAAD produced was an index of the LH-releasing activity of the extract. Dose-relationships have been obtained between the dose of hypothalamic extract given and OAAD (McCann, 1962; Ramirez & McCann, 1964; McCann, Schally, Nallar & Bowers, 1964).

Since in the OAAD assay the test cannot distinguish between LH-RF activity and any LH activity contaminating the sample, it is important to remove any LH from extracts by heat treatment which largely inactivates rat, ovine and bovine LH or by dialysis or gel

filtration which separate the two activities on the basis of molecular size (Guillemin, 1964). Of rather more importance in the assay is the report (Stevens, Jackson & Nalbandov, 1968) that OAAD was obtained with very large doses of porcine stalk-median eminence extract in hypophysectomised rats. The effect was not due to LH as it was not eliminated by filtration methods. Evidently, this extract contained a substance which depleted ovarian ascorbic acid by an extra-hypophysial mechanism (on the assumption that hypophysectomies were rigorously performed and assessed histologically). Since, for assay of LH-RF activity, the OAAD method depends on discharge of LH from the test animals anterior pituitary, it is worth noting that Novella, Alloiteau & Ascheim (1964) report that the pseudopregnant immature rat used in the OAAD assay has a pituitary LH concentration of only 2 μg/mg in comparison to the adult normal female rat in which the LH concentration is 10 μg/mg.

From the above remarks, it is evident that the OAAD assay must be interpreted cautiously. It will detect LH-RF activity, since samples active in this assay are also active in other in-vivo assays (Ramirez, Nallar & McCann, 1964; Schally, Bowers, White and Cohen, 1967) and in-vitro assays (Crighton, Watanabe, Dhariwal & McCann, 1968). In this laboratory it has been used (Fink, 1968; Fink & Harris, 1970; Harris & Ruf, 1970) for the detection of LH-RF activity in pituitary stalk blood. Bayman (1973) has used the OAAD assay to detect LH-RF in extracts of portal plasma from pro-oestrous rats.

A further type of in-vivo bioassay for LH-RF involves the injection of hypothalamic extracts into the ovariectomised oestrogen and progesterone treated rat (Ramirez & McCann, 1963). Increases in plasma LH follow the injection of as little as $^{1}/_{100}$th of a rat stalk-median eminence, whereas at least $^{2}/_{5}$ths of a rat stalk-median eminence is required in the direct OAAD assay.

In-vitro bioassays

The first report of the use of combined tissue cultures of adenohypophysial tissues and fragments of hypothalamus and median eminence was made by Guillemin and his co-workers (Guillemin, 1955a, 1955b; Guillemin & Rosenberg, 1955). The conclusion from these experiments was that pituitary explants survived in tissue culture for several weeks, and were capable of releasing ACTH into the culture medium on addition of hypothalamic extracts. Extracts of control tissues had no such effects, and from further pharmacological studies it was concluded that the substance involved was probably neither of the neurohypophysial octapeptides, nor was it epinephrine, histamine, acetylcholine, or serotonin. It seemed likely therefore, that the secretion of ACTH was due to the presence in hypothalamic extracts of some specific hypophysiotrophic mediator of unknown nature. Unsuccessful attempts were also made in these studies to stimulate secretion of thyroid stimulating hormone (TSH), growth hormone and

gonadotrophins (LH and FSH) by adding hypothalamic extracts to the culture fluids. This may have been due to destruction of hypothalamic-hypophysiotrophic substances by plasma, embryonic extract, or ascitic fluid included in the incubation medium as was later demonstrated in the case of TSH-RF by Redding and Schally (1968). Griffiths, Hooper & Hopkinson (1973) have demonstrated that rat hypothalamus itself is capable of inactivating LH-RF in-vitro.

Turning from tissue culture based assays, it is to the credit of Saffran & Schally (1955a) to have introduced the principle of one of the more elegant techniques for the study of the neurohumoral control of the secretion of the anterior pituitary gland by the hypothalamus. As described by them, the method involves the rapid removal of the anterior pituitary glands of rats, bisection at the isthmus and incubation of each individual hemipituitary in flasks containing oxygenated Krebs-Ringer bicarbonate glucose medium. The pituitary halves were incubated at 37° C for 60 minutes after addition of whatever was to be tested for ACTH releasing activity to one flask, and addition of control material to the other flask. At the end of the incubation period, the incubation fluids were assayed for ACTH activity using the in-vitro adrenal incubation system devised earlier also by Saffran and Schally (1955b).

The use of short term incubation of anterior pituitaries as a means of evaluating LH-RF activity was introduced in 1964 (Schally &

Bowers, 1964). Extracts of hypothalamus can increase the release of LH into the incubation medium (Schally & Bowers, 1964; Piacsek & Meites, 1966; Crighton, Watanabe, Dhariwal & McCann, 1968) whereas cerebral cortical extracts and extracts of hypothalamic regions outside the basal tuberal zone (Crighton & Schneider, 1969) are inactive. Dose-response relationships have been reported (Piacsek & Meites, 1966; Jutisz, Berault, Novella & Ribot, 1967) so that LH-RF can be quantitated by in-vitro assay. In-vitro stimulation by hypothalamic extracts of release of LH has been demonstrated with pituitaries of normal male (Piacsek & Meites, 1966) castrate male (Samli & Geschwind, 1967) and ovariectomised, oestrogen-progesterone treated female rats (Schally & Bowers, 1964; Samli & Geschwind, 1967). It is apparent that relatively more LH is released from glands from castrate animals than from those of normal males (Samli & Geschwind, 1967; Wakabayashi & Seldin, 1969).

The technique of in-vitro incubation of rat pituitaries in the study of neurohumoral mechanisms involved in the control of the secretion of pituitary hormones can be considered as a specific, reliable and sensitive method. It has disadvantages however. To be secure about its conclusions, any one experiment should ideally use identical populations of hemipituitaries, one flask of hemipairs serving as control for the other flask of corresponding hemipairs. In other words, each incubation of two beakers can study only one

experimental variant. What this means is that it is not strictly correct to randomise a number of pituitaries or hemipituitaries placing them in more than two beakers adding different treatments to these beakers to compare the effects of the different treatments. If this design restriction is adhered to however, the usefulness of the technique for establishing dose-response curves and reliable estimates of the potency of experimental materials is obviated.

One could envisage that if one were to use a very large number of pituitaries, placing them in multiple beakers one might, minimise or statistically eliminate any inherent variability in the responsiveness of each individual pituitary. The cost of such a design would be prohibitive. Another approach would be to use anterior pituitary glands from animals sufficiently large to provide tissue for many incubation flasks from the same gland (La Bella, 1964). With this sort of design ready availability and freshness of tissue might prove a problem.

Despite the difficulties discussed above the experiments to be described employ an experimental design in which hemipituitaries from a homogeneous population of animals were randomised amongst a relatively large number of incubation flasks. It seemed that the only way to establish dose-response curves and to compare treatments within one assay was to proceed in this way. Clearly, however, the

interpretation of the results must be made against a background in which the inherent limitations of the method are weighed carefully against its advantages in terms of cost, and relative simplicity, and in which information gained from other forms of assay is used for purposes of comparison.

The great advantage of in-vitro bioassays is that they are systems in which the effects of endogenous materials can be discounted. Endogenous materials may often complicate the assay of hypophysiotrophic substances in-vivo. However, in-vitro bioassay findings should wherever possible be confirmed in in-vivo systems. Any substance of hypothalamic origin active in-vivo on the release of adenohypophysial hormones should also be active in-vitro. The reverse is not necessarily true.

As has been pointed out, previous studies in this laboratory on LH-RF activity of pituitary stalk blood were carried out with the aid of in-vivo assays. Further work on this problem required a sensitive and specific method which would permit the assay of large numbers of samples, and in which the response was a linear function of the dose. It seemed that an in-vitro assay based on the method first described by Saffran & Schally (1955a)might best meet these requirements. The majority of short term incubation assay methods published have used pituitary glands from male rats, and relatively many glands (3 - 12) in each incubation flask (Mittler & Meites, 1966;

Guillemin & Vale, 1970; McCann, 1970; Schally, Redding & Arimura, 1973). Relatively few workers (Schally & Bowers, 1964; Samli & Geschwind, 1967; Jutisz, Kerdelhue & Berault, 1970) have employed pituitary glands from a preparation which is highly sensitive to LH-RF activity, the ovariectomised rat treated with oestrogen and progesterone (OOP rat, Ramirez & McCann, 1963). In order to select the optimal and most economical method comparison has been made between the characteristics of an assay system using 3 - 4 male pituitary glands/flask with those of a system using pituitary glands from OOP rats, 1 gland/flask. The latter system has been applied in the first instance to the investigation of the activity of "fragments" of LH-RF, and of a tripeptide (Igarashi, 1971) and a tetrapeptide (Chang, Sievertsson, Bogentoft, Currie, Folkers & Bowers, 1971) which are said to have luteinising-hormone releasing activity.

3.4. Experimental methods

Animals

Adult male and female rats of the Wistar strain (Charles River (UK) Ltd., Margate) weighing 250 -350 g were used as donors of anterior pituitary glands. Female rats which had been ovariectomised by bilateral flank incisions under ether anaesthesia at least three weeks before were injected s.c. with 25 mg progesterone

in almond oil, and 50 µg oestradiol benzoate in 20 % ethyl oleate and 80 % arachis oil (Evans Medical Ltd., Liverpool) 72 hours prior to assay. All animals were maintained under conditions of controlled light (14 h light, 10 h dark) and temperature (22° C) and fed diet 41 B pellets (E. Dixon & Sons Ltd., Ware) and tap water ad libitum.

Assay procedure

Assays (method reported in preliminary form by Jamieson, Fink & Chiappa, 1973) were carried out using either male pituitary glands (6 - 8 pituitary halves/incubation flask) or female pituitary glands (2 pituitary halves/incubation flask). In each case the animals were anaesthetised with sodium pentobarbitone (Expiral, Abbot Laboratories; Kent) and decapitated. The anterior pituitary glands were removed, bisected longitudinally and placed in 25 ml siliconised glass flasks containing 2 ml of Minimum Essential Medium with Earles Salts and 20 mM Hepes Buffer (Flow Laboratories; Ayrshire), to which one drop of 20 mM L-glutamine had been added (see Appendix 3.1).

Preincubation was carried out for 1 hour at 37° C under 95 % O_2 and 5 % CO_2 in a shaking incubator (Gallenkamp) shaking at 70 c/min. The medium was then aspirated, discarded, and replaced with fresh medium. Two or three doses of synthetic (vide infra) LH-RF (0.5 - 33.0 ng/ml medium and 1.9 to 30.0 ng/ml medium in male and female assay systems respectively) which was used as a standard, and

one or two doses of materials under investigation were added to incubation flasks in a volume of 300 µl distilled water. Control flasks received 300 µl of distilled water. Incubation flasks were randomised using Tables of Random Numbers (Fisher & Yates, 1963) into groups of four flasks per dose. Incubation was carried out for 6 hours, after which samples of the medium were stored at -15° C. In earlier assays the glands were blotted and weighed at the end of incubation. On occasions glands were fixed immediately after assay for light microscopy with Formol-sublimate. A flow chart of the assay appears in Figure 3.2.

Synthetic LH-RF

The synthetic LH-RF, fragments of LH-RF and the tri- and tetrapeptides used in this study were synthesised by Drs. H. Gregory, J. Gormley and A.M. Rowbotham (I.C.I. Pharmaceuticals, Macclesfield), and stored at a concentration of 1 mg/ml of distilled water at -15°C (Appendix 3.2).

Assay of LH and FSH

The end-point of the assays was the increase produced in the LH and FSH concentrations of the medium after 6 hours of incubation.

The LH content of medium was assayed by the ovine-ovine double antibody radioimmunoassay method developed by Niswender,

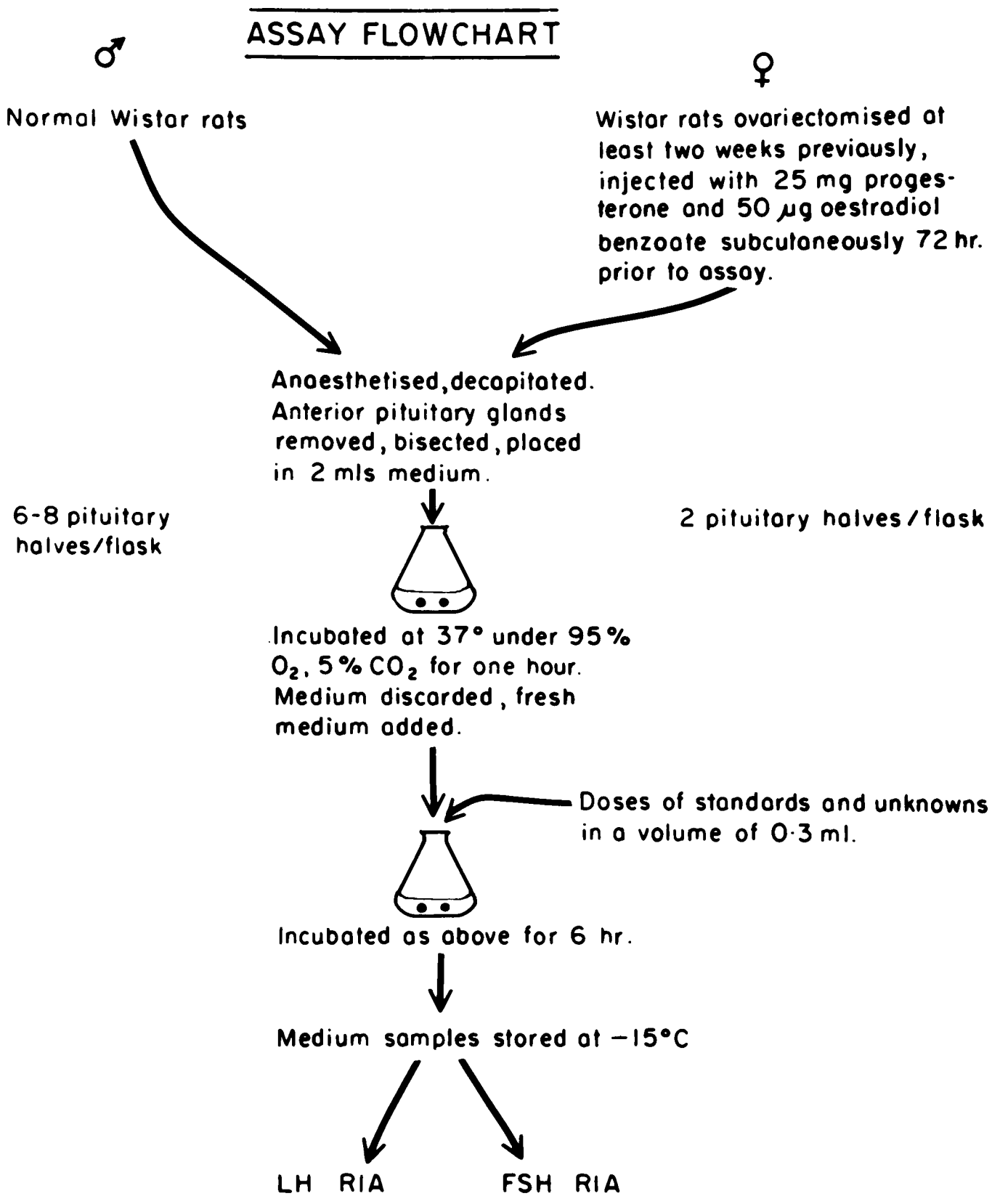


Figure 3.2 Flowchart of in-vitro bioassays for LH-RF.

Midgely, Monroe & Reichert (1968) and the results are expressed as ng of the ovine standard (NIH-LH-S 13) per ml medium. The FSH content of medium was assayed by the NIAMD rat FSH radioimmunoassay kit; results are expressed as ng of the standard NIAMD-rat-FSH-RP 1 per ml medium. In both assays, samples were diluted 500 - 1000 fold with 1 % egg white/phosphate buffered saline, and then assayed in duplicates of 200 µl/assay tube.

In both the LH (Figure 3.3) and FSH (Figure 3.4) radioimmunoassays the dose-response curves of the incubation medium and NIH-LH-S 13 and NIAMD-rat-FSH-RP 1 respectively were parallel.

Statistical Treatment

The significance of differences between groups was determined by Students t test: $p < 0.05$ being taken as the level at which differences were significant. Calculation of regression equations, precision, potencies and confidence limits was made according to Bliss (1952).

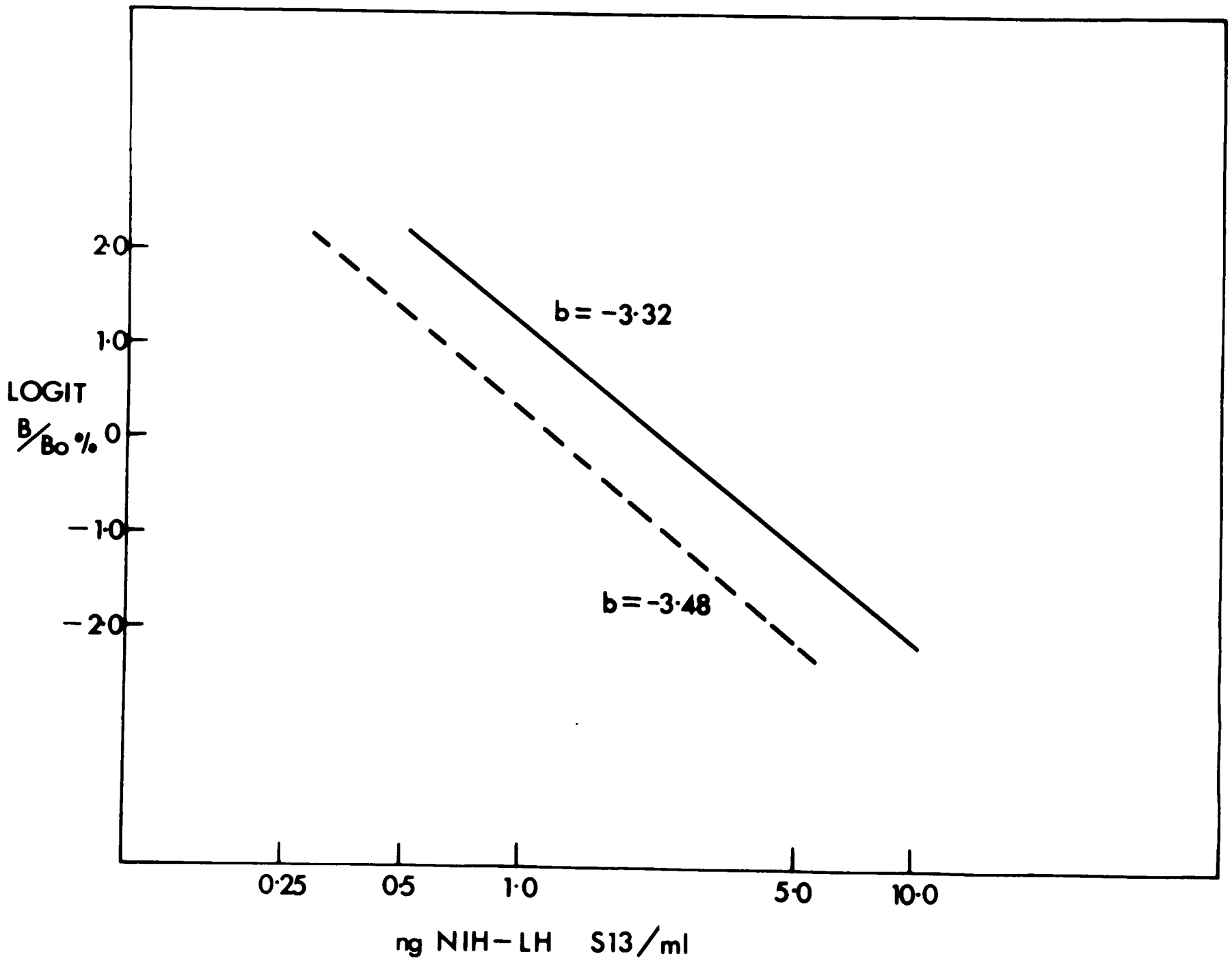
3.5. RESULTS

Effect of synthetic LH-RF on the release of LH and FSH

Male system

Incubation of 6 - 8 male pituitary halves with two or three

Figure 3.3 Inhibition curves for standard LH, and for incubation medium containing LH, in LH RIA.
(——) (- - - -)



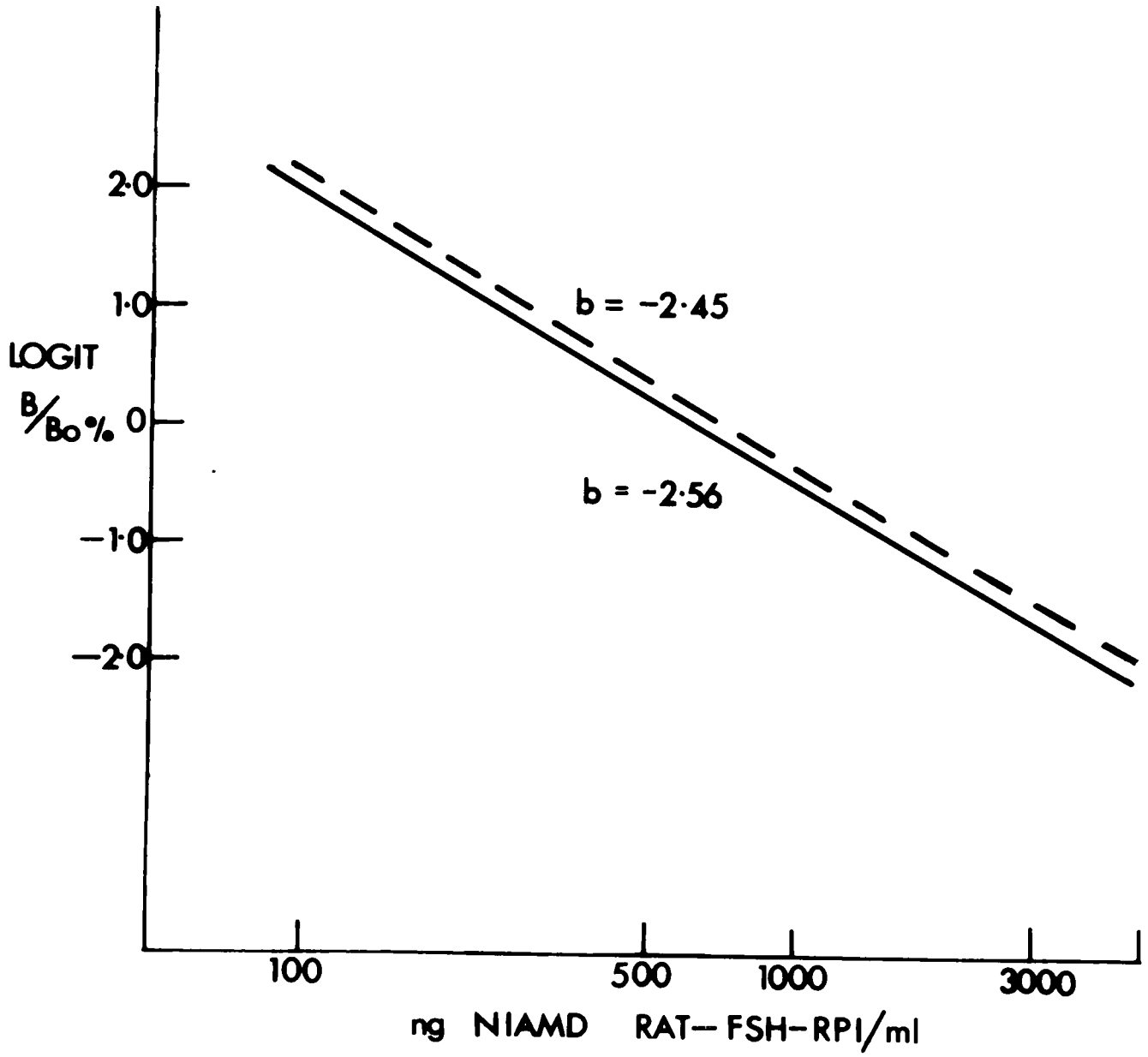


Figure 3.4 Inhibition curves for standard FSH, and for incubation (——) medium containing FSH, in FSH RIA. (----)

doses of LH-RF promoted LH and FSH release which was significant when compared to controls in six independent assays. The system was consistently sensitive to a concentration of 8.0 ng LH-RF/ml medium, and, in three assays, to a concentration of 2.0 ng LH-RF/ml medium (Table 3.1). The precision (λ) of these assays (4 flasks/dose) with respect to LH release varied from 0.15 to 0.28 (0.20 $\pm$ 0.02, Mean $\pm$ S.E.M.). With respect to FSH release, λ ranged from 0.13 to 0.31 (0.23 $\pm$ 0.03). The slopes of the calculated regression lines varied from 979 to 2108 (1490 $\pm$ 169) for LH release (Figure 3.5) and from 9500 to 23810 (16106 $\pm$ 2623) for FSH release (Figure 3.6). The correlation coefficient between the slopes for LH and FSH release was 0.69 ($p > 0.1$). ~~and the slope ratio between the LH and FSH dose-response lines was 0.099 + 0.006.~~

When potassium chloride solution was added to incubation flasks it was added to give final concentrations of 6.7, 13.2 and 30.5 mM K^+. Only the highest concentration of potassium had any significant effect on release of LH or FSH (Table 3.2).

The weight of pituitary tissue in incubation flasks at the end of incubation in five assays was 16.8 $\pm$ 0.3 mg/flask (n = 116).

Female system

Incubation of two pituitary halves from OOP rats with three

ASSAY NO.	CONTROL	DOSE LH-RF ng/ml medium				b	λ
		0.5	2.0	8.0	33.0		
1. LH ng/ml medium	**	700	1622	2606	-	1602	0.16
FSH ng/ml medium	**	11720	18830	23840	-	9940	0.14
2. LH	**	418	615	1203	-	979	0.18
FSH	**	10650	11620	17330	-	9500	0.21
3. LH	491	-	*926	1743	3442	1326	0.23
FSH	7360	-	*11500	17800	36380	11080	0.28
4. LH	653	-	517	*1379	2445	1757	0.21
FSH	8450	-	9570	*16480	28580	21010	0.13
5. LH	503	-	-	*871	1587	1169	0.15
FSH	9970	-	-	*14770	21850	11650	0.31
6. LH	710	-	470	*1190	2460	2108	0.28
FSH	11830	-	8930	*18530	32830	23810	0.30
MEAN ± S.E.M.							
LH	589 ± 55	559 ± 141	830 ± 213	1498 ± 250	2483 ± 380	1490 ± 169	0.20 ± 0.02
FSH	9402 ± 970	11185 ± 535	12090 ± 1766	18065 ± 1204	29910 ± 3124	16106 ± 2623	0.23 ± 0.03
				p<0.05	p<0.01		

TABLE 3.1 The effect of synthetic LH-RF on LH and FSH release from male rat anterior pituitary glands incubated in-vitro (4 flasks/dose).

* $p < 0.05$ when compared with controls.

** No controls included in early assays.

Figure 3.5

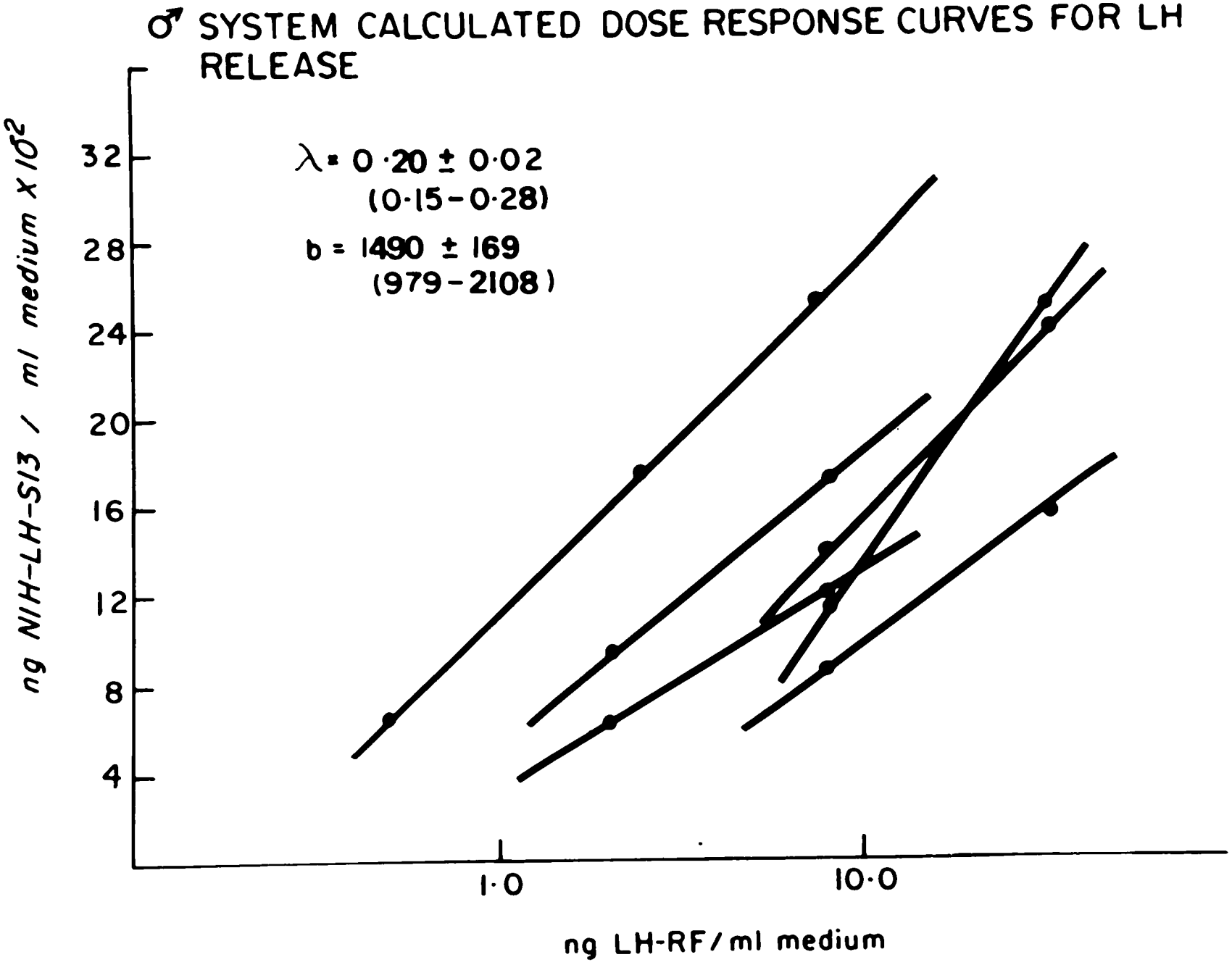
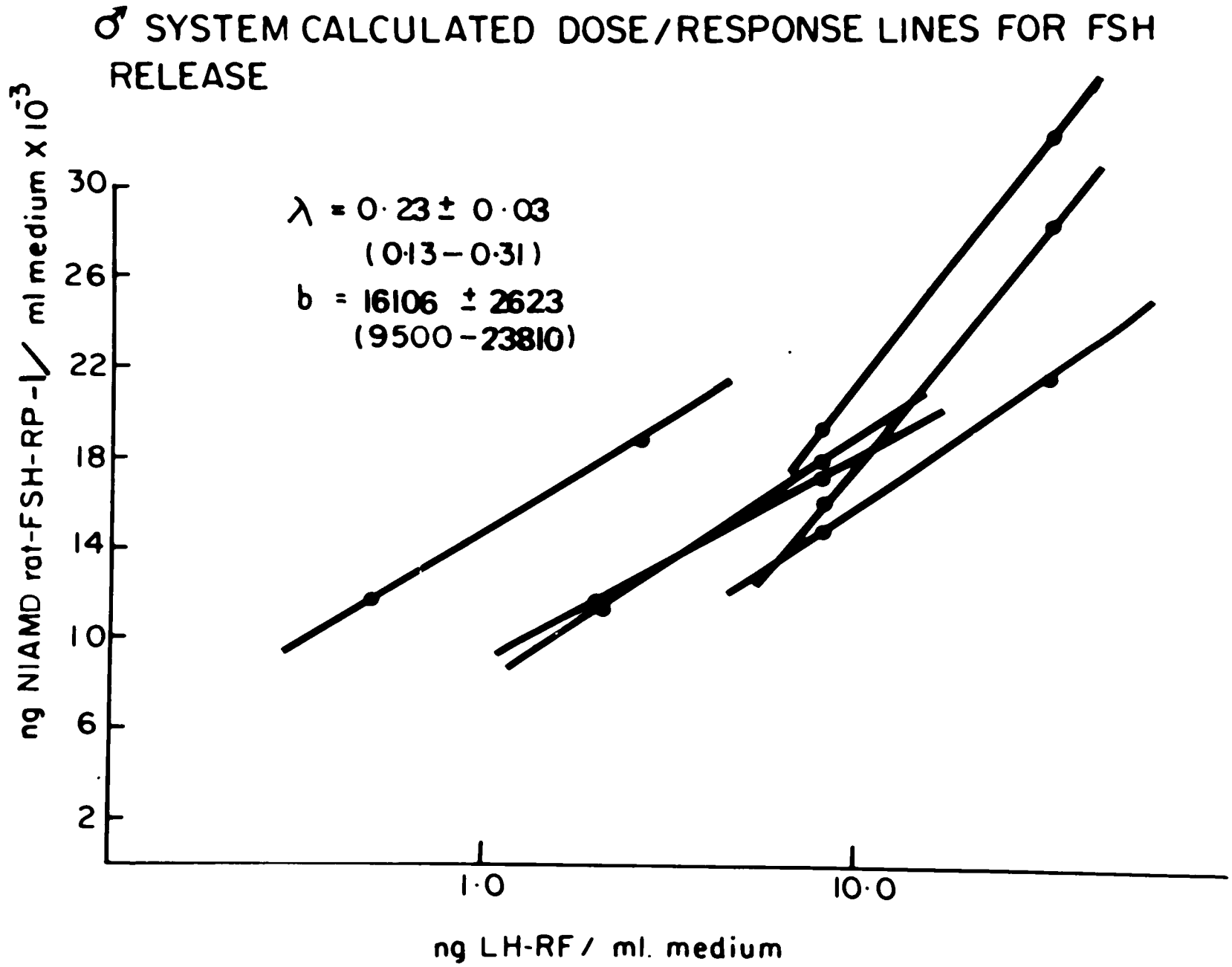

♂ SYSTEM CALCULATED DOSE RESPONSE CURVES FOR LH RELEASE
λ = 0·20 ± 0·02
(0·15-0·28)
b = 1490 ± 169
(979-2108)
ng NIH-LH-S13 / ml medium X 10²
32
28
24
20
16
12
8
4
1·0
10·0
ng LH-RF / ml medium

Figure 3.6


♂ SYSTEM CALCULATED DOSE/RESPONSE LINES FOR FSH RELEASE
λ = 0·23 ± 0·03
(0·13 – 0·31)
b = 16106 ± 2623
(9500 – 23810)
ng NIAMD rat-FSH-RP-1/ ml medium X 10⁻³
30
26
22
18
14
10
6
2
1·0
10·0
ng LH-RF / ml. medium

	CONTROL	LH-RF ng/ml medium		m Molarity K^+		
		8.0	33.0	6.7	13.2	30.5
LH ng/ml medium	503 ± 38	* 871 ± 26	* 1587 ± 146	641 ± 101	627 ± 74	* 1283 ± 86
FSH ng/ml medium	9970 ± 950	* 14770 ± 1030	* 21850 ± 2360	10720 ± 710	10800 ± 1170	* 15250 ± 1480

* $p < 0.05$ when compared with saline control.

TABLE 3.2 Male assay system: effect of various concentrations of K^+ on release of LH and FSH (mean ± S.E.M., 4 flasks/dose).

doses of LH-RF promoted LH and FSH release which was significant when compared to controls in fourteen independent assays. This system was generally sensitive to a concentration of 1.9 ng LH-RF/ml medium (Table 3.3). The precision of these assays with respect to LH release varied from 0.09 to 0.34 (0.19 $\pm$ 0.02). With respect to FSH release the precision varied from 0.04 to 0.35 (0.19 $\pm$ 0.02). The slopes of the calculated regression lines varied from 2780 - 10330 (5813 $\pm$ 535) for LH release (Figure 3.7) and from 9260 - 27000 (15422 $\pm$ 1772) for FSH release (Figure 3.8). The correlation coefficient between the slopes for LH and FSH release was 0.45 ($p > 0.1$). ~~and the slope ratio between the LH and FSH dose-response lines was 0.436 $\pm$ 0.072~~.

A concentration of 13.2 mM K^+ in medium had no significant effect on release of LH and FSH; however, a significant amount of LH and FSH was released when pituitaries were incubated in medium containing 30.5 mM K^+ (Table 3.4).

When epinephrine hydrogen tartrate, L-nor-epinephrine (BDH; Dorset) or dopamine (Sigma; London) were added to incubation flasks to give final concentrations of 50 mg/l medium, there was no significant effect on release of either LH or FSH (Table 3.5). When oxytocin or vasopressin (Parke Davis; USA) were added to incubation flasks to give final concentrations of 100 U/l medium,

TABLE 3.3

ASSAY NO.	CONTROL	DOSE LH-RF ng/ml medium			b	λ
		1.9	7.5	30.0		
7. LH ng/ml medium	810	1168	*2492	5457	5081	0.20
FSH ng/ml medium	4230	4730	*8730	14300	9530	0.18
8. LH	1210	1385	*2505	4185	2815	0.20
FSH	4880	*5950	8430	16870	14240	0.23
9. LH	940	*3660	6150	7800	4090	0.34
FSH	4710	*11730	18550	20020	11220	0.21
10. LH	560	*1305	5875	6300	7525	0.11
FSH	3320	*5156	13150	15940	13140	0.17
11. LH	653	*3455	6000	-	8595	0.12
FSH	4320	*10600	15030	-	21250	0.04
12. LH	810	*2945	5200	7500	3750	0.27
FSH	3990	*10330	13780	23250	10700	0.13
13. LH	750	*2440	3310	6650	5615	0.28
FSH	3460	*9900	11950	20000	22430	0.10
14. LH	1150	*4650	8175	9440	5810	0.21
FSH	3930	*10580	13670	16330	4410	0.35
15. LH	1330	*2420	6010	7940	5890	0.19
FSH	5780	7150	*12830	19450	11046	0.25
16. LH	1050	*5250	7180	7450	3174	0.09
FSH	5000	*16200	22900	25060	11050	0.28
17. LH	870	*3050	4950	11150	10330	0.16
FSH	3460	*8800	13800	29830	26720	0.15

ASSAY NO.	CONTROL	DOSE LH-RF ng/ml medium			b	λ
		1.9	7.5	30.0		
18. LH	1180	*5540	6470	10350	6470	0.19
FSH	5280	*15200	19800	25940	10230	0.19
19. LH	1050	*4590	7410	6760	4600	0.16
FSH	6400	*16200	26000	28100	16120	0.30
20. LH	1580	*5820	5750	9230	5790	0.25
FSH	6850	*17800	20800	37000	27000	0.16
21. LH	1220	1710	*2810	7420	7660	0.09
FSH	5910	7580	*11890	25250	22250	0.14
MEAN						
+ S.E.M.						
LH	1011	3292	5352	7688	5813	0.19
	± 70	± 410	± 467	± 502	± 535	± 0.02
		p<0.01	p<0.01			
FSH	4768	10527	15420	22667	15422	0.19
	± 284	± 1086	± 1315	± 1694	± 1772	± 0.02
		p<0.01	p<0.01			

TABLE 3.3 The effect of synthetic LH-RF on LH and FSH release from female rat anterior pituitary glands incubated in-vitro (4 flasks/dose).

* $p < 0.05$ when compared with controls.

. . . . ./NOTE

NOTE. In Assay II, a dose of 3.75 ng L.H-RF/ml medium was included, the response for LH being 3750 ng/ml medium and that for FSH being 9530 ng/ml medium. This dose, and that at 7.5 ng/ml medium were used for calculation of the regression lines.

Figure 3.7

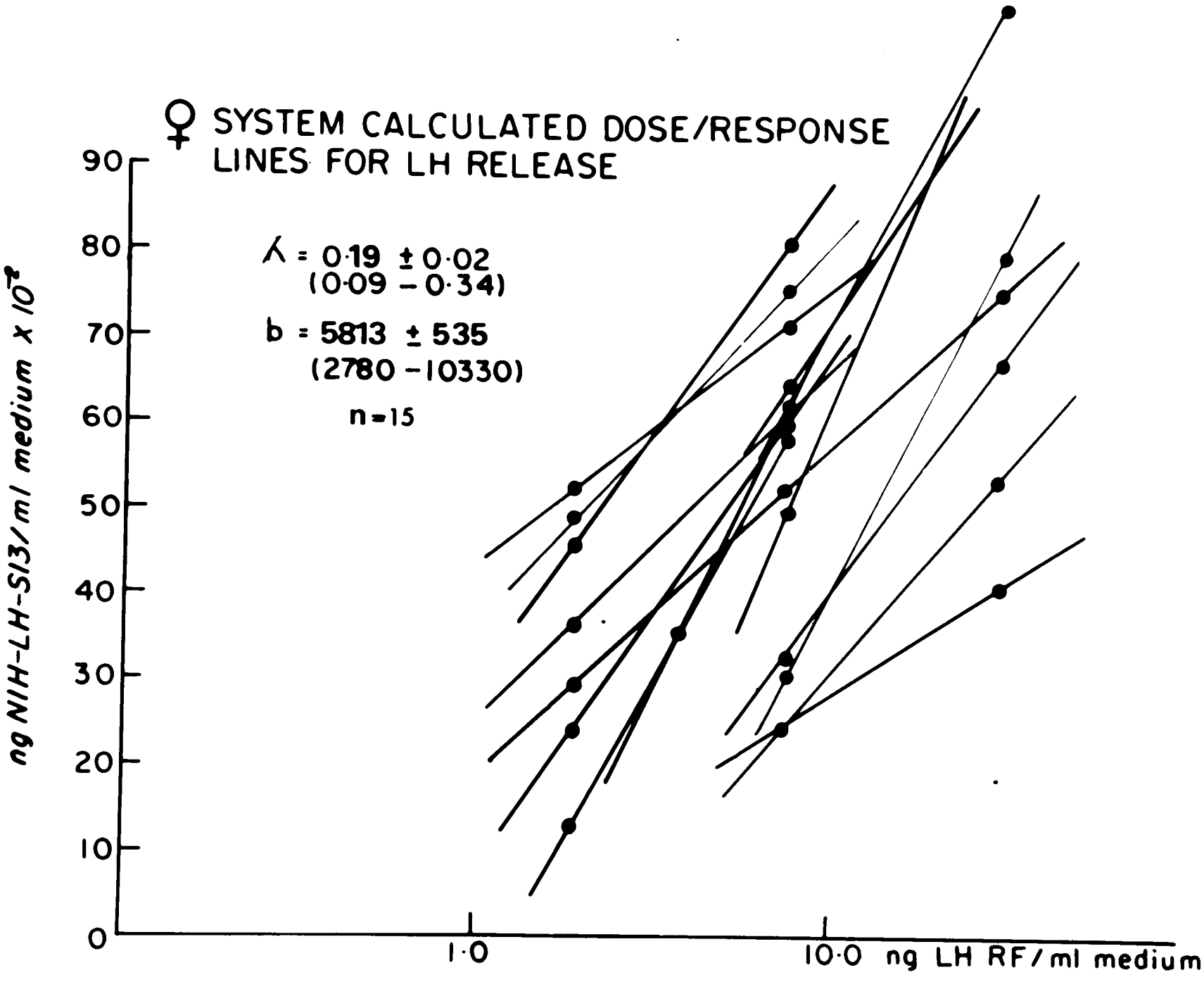
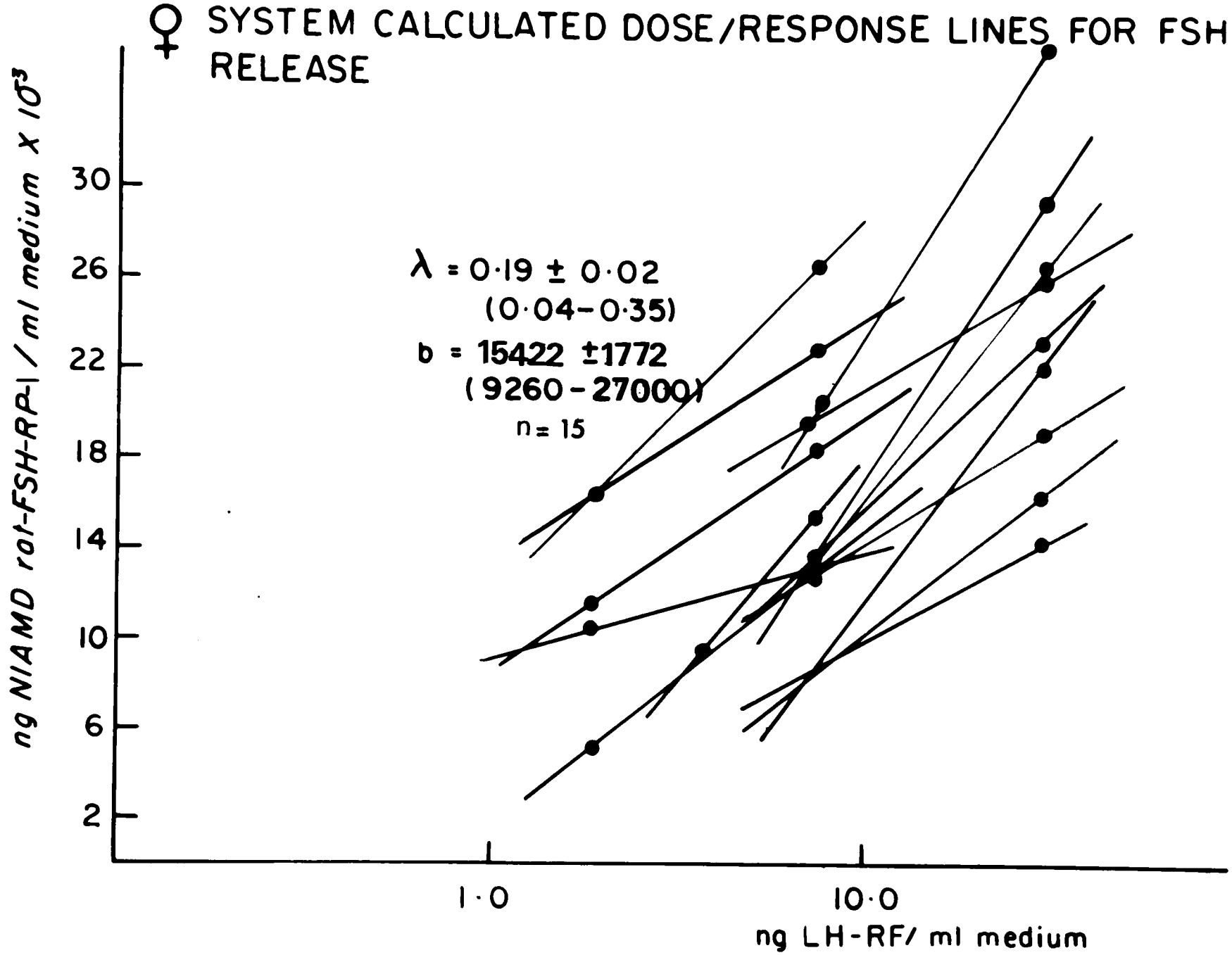

♀ SYSTEM CALCULATED DOSE/RESPONSE LINES FOR LH RELEASE
λ = 0·19 ± 0·02 (0·09 – 0·34)
b = 5813 ± 535 (2780 – 10330)
n = 15
ng NIH-LH-S13/ml medium x 10⁻²
90
80
70
60
50
40
30
20
10
0
1·0
10·0 ng LH RF/ml medium

Figure 3.8


♀ SYSTEM CALCULATED DOSE/RESPONSE LINES FOR FSH RELEASE
λ = 0·19 ± 0·02
(0·04–0·35)
b = 15422 ±1772
(9260–27000)
n = 15
ng NIAMD rat-FSH-RP-1 / ml medium X 10³
30
26
22
18
14
10
6
2
1·0
10·0
ng LH-RF/ ml medium

	CONTROL	LH-RF ng/ml medium		m Molarity K^+	
		1.9	7.5	13.2	30.5
LH ng/ml medium	1330 ± 240	*2420 ± 80	*6010 ± 690	2490 ± 510	*6670 ± 290
FSH ng/ml medium	5780 ± 680	7150 ± 400	*12830 ± 1520	6580 ± 990	*13530 ± 510

* $p < 0.05$ when compared with saline control.

TABLE 3.4 Female assay system: effect of various concentrations of K^+ on release of LH and FSH (mean ± S.E.M., 4 flasks/dose).

vasopressin, but not oxytocin, had a slight stimulatory effect on the release of both LH and FSH (Table 3.5).

The weight of pituitary tissue in incubation flasks at the end of incubation in six assays was 13.6 $\pm$ 0.2 mg/flask (n = 220).

Histology

Paraffin sections cut at 6 µm were stained either with haematoxylin and eosin or with the Periodic Acid/Schiff histochemical method.

At low power, sections through hemipituitaries demonstrated that while the adenohypophysial cells in the outer third of the glands appeared to be normal, there was considerable loss of cell definition in the central two thirds, with pyknotic nuclei, large intercellular gaps and cytoplasmic fragmentation. In the outer third, there was frequent magenta staining of gonadotrophs. Photomicrographs of a section stained with haematoxylin and eosin appear in Figure 3.9.

Activity of synthetic fragments of LH-RF and other peptides

Nine synthetic peptides (Appendix 3.2) were tested for LH and FSH releasing activity in the female assay system. These peptides were tested in the assay at dosages ranging from 30 to 2160 ng/ml medium. In several assays, only the nonapeptide consistently released

	CONTROL	LH-RF ng/ml medium		Adrenaline	Noradrenaline	Dopamine	Oxytocin	Vasopressin
		8.0	33.0		50 mg/l medium		100 u/l medium	
LH ng/ml medium	1180 ± 190	*6470 ± 590	*1035 ± 530	820 ± 80	810 ± 130	1320 ± 210	990 ± 140	*2110 ± 240
FSH ng/ml medium	5280 ± 20	*19800 ± 1160	*2594 ± 690	5100 ± 300	4820 ± 130	5450 ± 940	5250 ± 410	*7250 ± 370

* $p < 0.05$ when compared with control

TABLE 3.5 Female assay system: effect of adrenaline, nor-adrenaline, dopamine, oxytocin and vasopressin on LH and FSH release (mean ± S.E.M., 4 flasks/dose).

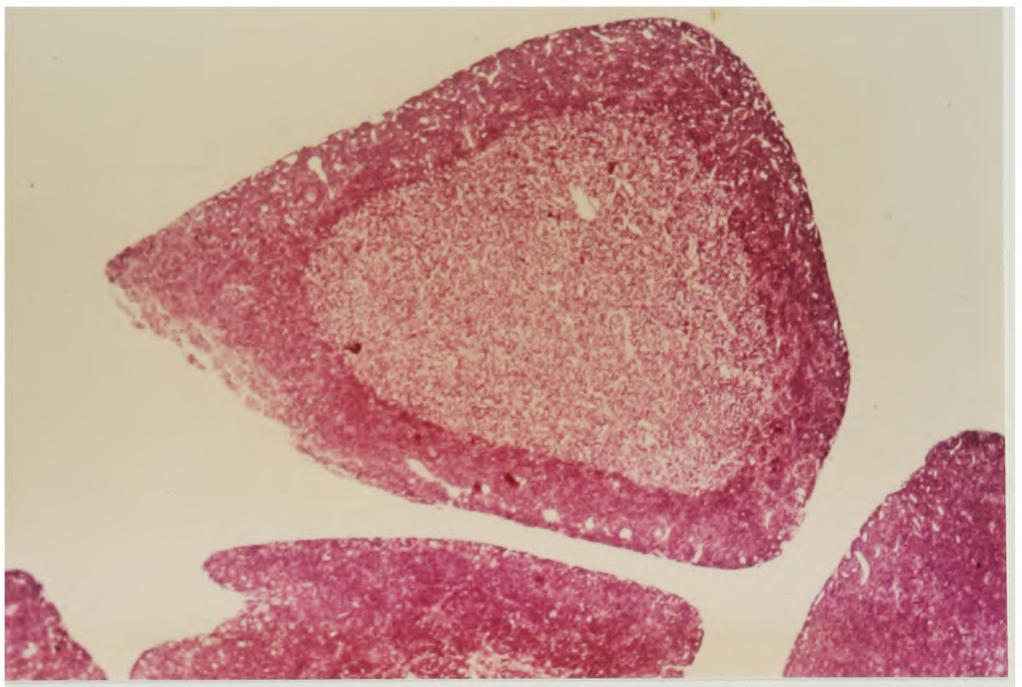


Figure 3.9

Plate. Photomicrograph of H + E stained section of female assay system hemipituitary.

LH and FSH. In two independent assays two doses of the nonapeptide, 30 and 135 ng/ml medium were included. With respect to LH release, the dose response curves of the nonapeptide did not diverge significantly from those of the decapeptide (Figure 3.10), the potency (95 % confidence limits) of the nonapeptide being 7.6 (1.8 - 15.5) and 17.8 (6.2 - 35.5) ng in terms of LH-RF in the first and second assays respectively. With respect to FSH release a dose-response curve which did not diverge significantly from that of the decapeptide was detained in only one assay (Figure 3.11) the potency being 3.6 (1.1 - 7.1) ng in terms of the decapeptide. In the second assay divergence between the slopes of the dose-response curves was significant ($F = 19.2$); using a 2 + 1 calculation, the upper dose of the nonapeptide released an amount of FSH equivalent to 11.7 ng LH-RF. The worksheets for the assays in which dose response curves parallel to those of the standard preparation were obtained for LH and FSH release appear in Appendix 3.3.

Very slight degrees of activity were exhibited by the -N and -C terminal octapeptides (LH-RF 1 - 8 and LH-RF 3 - 10) but dose response curves could not be obtained. A summary of the results appears in Table 3.6.

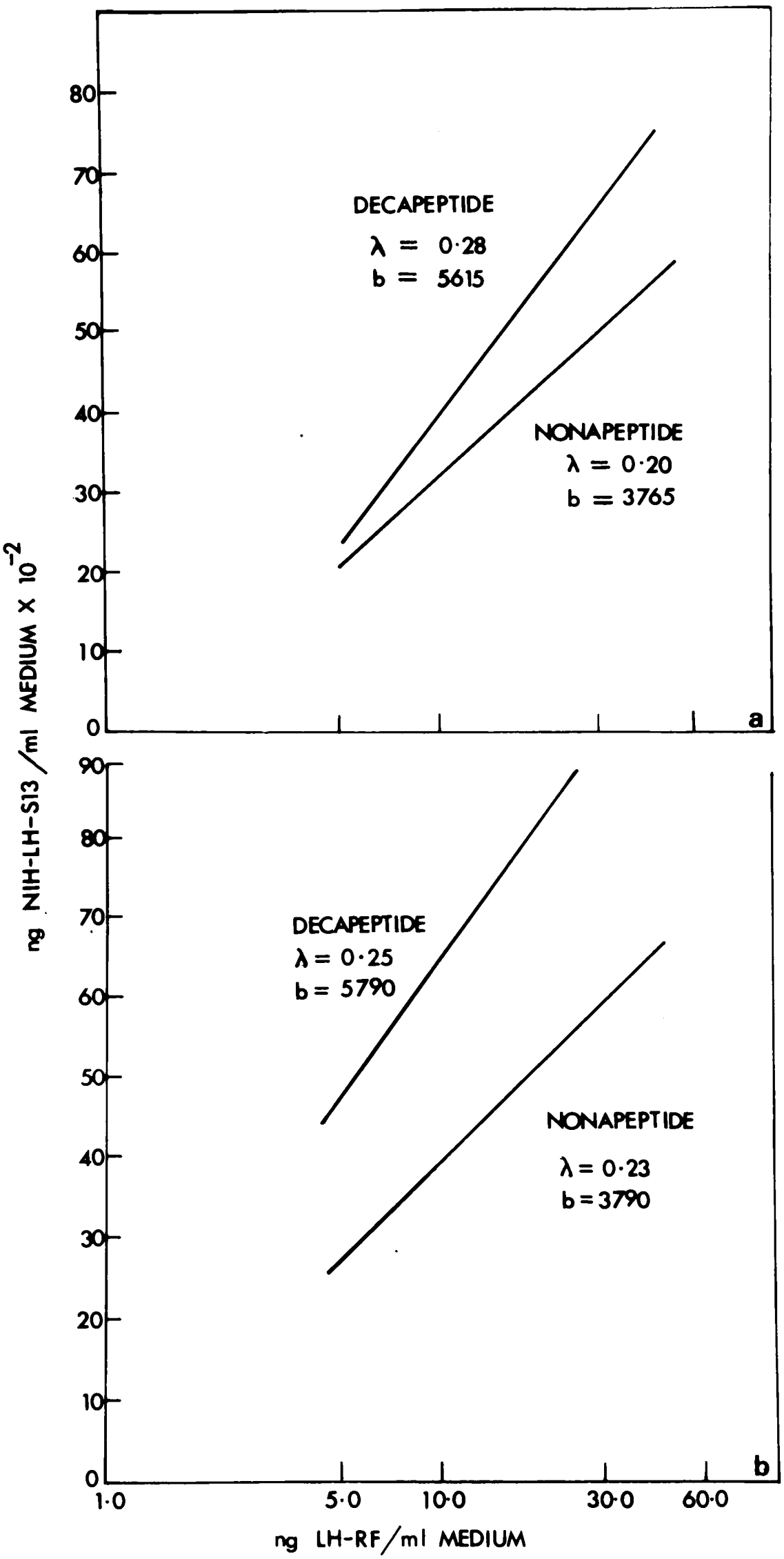


Figure 3.10 Dose-response curves for synthetic LH-RF (Standard, decapeptide) and for LH-RF 1-9 (nonapeptide) in Assays No 13 (a) and 20 (b).

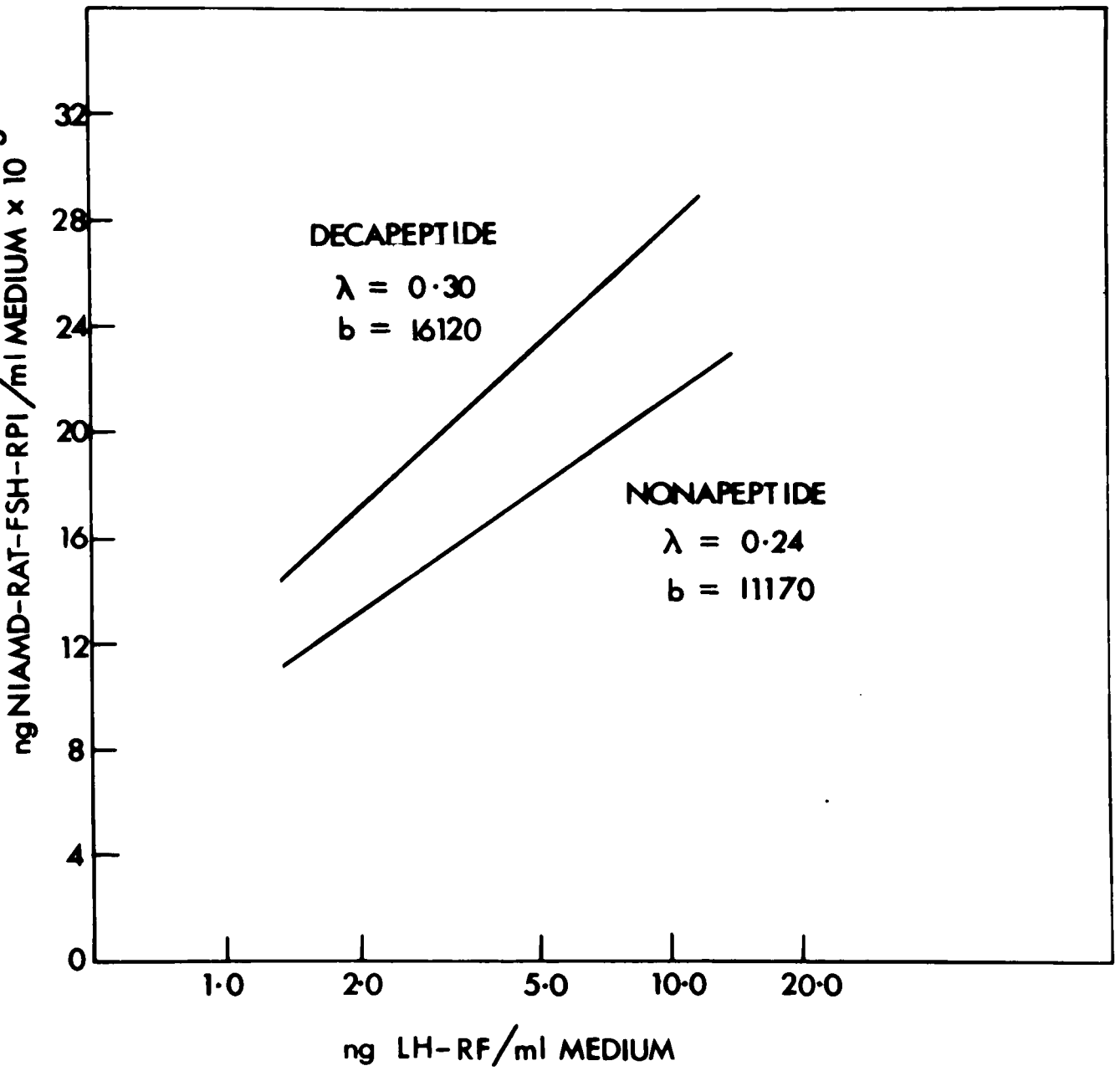


Figure 3.11 Dose-response curves for synthetic LH-RF (Standard, decapeptide) and for LH-RF 1-9 (nonapeptide) in Assay No 19.

TABLE 3.6 POTENCY OF SYNTHETIC PEPTIDES IN TERMS OF DECAPEPTIDE (LH-RF) FOR LH AND FSH RELEASING ACTIVITY.

SYNTHETIC PEPTIDE	DOSE ng/ml medium							
	30		135		540		2160	
	LH	FSH	LH	FSH	LH	FSH	LH	FSH
F_1 (Igarashi)	-	-	NS	NS	-	-	-	-
F_2 (Folkers)	-	-	NS	NS	-	-	-	-
F_3 (LH-RF 1-9)			7.6** (1.8-15.5)	3.6** (1.1-7.1)				
	5.0*	6.2*	17.8** (6.2-35.5)	11.7*				
F_4 (LH-RF 1-8)	NS	NS	1.6†	NS	NS	4.9†	NS	NS
F_5 (LH-RF 1-6)	NS	NS	NS	NS	NS	NS	-	-
F_6 (LH-RF 1-2+9-10)	NS	NS	NS	NS	NS	NS	-	-
F_7 (LH-RF 3-10)	NS	NS	1.8†	NS	-	-	-	-
F_8 (LH-RF 3-9)	-	-	NS	NS	NS	NS	-	-
F_9 (LH-RF 7-10)	-	-	NS	NS	NS	NS	-	-

NS = not significantly different from saline.

* Mean potency in terms of ng decapeptide (LH-RF) /ml medium - one dosage level of synthetic peptide

** Mean potency (95 %) confidence limits) in terms of ng decapeptide (LH-RF)/ml medium - two dosage levels of synthetic peptide

† Potency obtained in one 2+1 assay but not confirmed in subsequent assays.

3.6. DISCUSSION

The first in-vitro assays which used pituitary glands from OOP rats to detect LH and FSH releasing activity (Schally & Bowers, 1964; Kuroshima, Ishida, Bowers & Schally, 1965) used large amounts of pituitary tissue (7 - 24 hemipituitaries/flask) presumably because bioassays were used to determine gonadotrophin release. Subsequently Samli & Geschwind (1967) and Jutisz, Kerdelhue & Berault (1970), who determined the concentration of LH in the incubation medium by immunoassay methods used 1 - 6 and 4 - 5 pituitary halves/flask respectively. The present study shows that with respect to the assay of the LH-RF decapeptide, a system using one OOP rat pituitary gland per flask has a sensitivity comparable to that of a system using 3 - 4 (present study) or 5 (Schally, Redding, Matsuo & Arimura, 1972) male glands per flask. Precision of the female system is similar to that of the male system. It is important to point out that in establishing the validity of the in-vitro assay described here, it was shown that there was no significant divergence between the slope of the dose response curve of successive dilutions of the incubation medium (containing both LH and FSH) and the slope of the standard curve in both the LH and FSH radioimmunoassays. Apart from Schally and Bowers (1964) and possibly Jutisz, Kerdelhue & Berault (1970), this important point has received little attention from workers using either male or female pituitary glands in in-vitro assay systems for

LH-RF.

The principal desirable characteristics of an assay method are specificity, precision, low dose requirement or sensitivity, repeatability, convenience and economy.

In so far as it has been tested the female assay provides good specificity. Of the monoamines and octapeptides that might be expected to be found in extracts of brain tissues, or in pituitary portal plasma extracts, only vasopressin caused significant release of LH and FSH and that at a dose (100 U/l) vastly in excess of the levels of vasopressin that would be expected even in extracts of the posterior pituitary alone. In the dissection of pituitary glands for use in the present assay systems, the posterior pituitary (which in the rat is easily separated from the anterior lobe at the convenient plane of the intermediate lobe) is removed prior to bisection of the gland. Conceivably, small amounts of posterior lobe tissue might be transferred to incubation flasks during this procedure. Synaptic transmitters such as acetylcholine, gamma-amino butyric acid, and serotonin and histamine have not been tested for activity. Histamine and serotonin have no effect on in-vivo bioassay using OAAD as the index of LH secretion (McCann, Taleisnik & Friedman, 1960).

It is clear that high concentrations of potassium can cause the release of LH and FSH from anterior pituitary glands incubated

in-vitro. Samli and Geschwind (1968) found that a ten fold increase in K^+ concentration (from 5.9 mM to 59 mM) markedly increased release of LH. Wakabayashi and McCann (1970) found that a K^+ concentration of 60 mM released LH from normal and castrated male pituitary glands in-vitro. In the present study, concentrations of K^+ of 30.5 mM were sufficient to cause release of LH and FSH in both male and female systems. The concentration of K^+ in the incubation medium is 5.2 mM, and therefore there is a considerable safety margin with respect to potassium. At the end of incubation, potassium concentration in incubation flasks was 5.0 - 6.0 mM. The influence of cyclic AMP and of prostaglandins has not been investigated in the present studies. Cyclic AMP has been reported to cause release of LH from incubated rat pituitaries (Jutisz, Kerdelhue & Berault, 1970) and prostaglandin E_2 (Vale, Grant, Amoss, Blackwell & Guillemin, 1972) releases TSH from cultures of anterior pituitary cells.

The precision of an assay, λ, is derived from the ratio of the standard deviation, S, of the dose-response curve, to the slope, b, of the linear portion of the dose-response curve. The lower the value of λ, the more precise the assay. There is a consensus among assay statisticians (Bliss, 1952) that values of λ below the arbitrary value of 0.3 are acceptable, and in the male and female assay systems described, mean values for λ appear satisfactory, although there is considerable variation.

The minimum dose of synthetic LH-RF tried in the female assay system was 1.9 ng/ml medium which was effective in eleven of fourteen assays. The male system consistently responded to 8.0 ng/ml medium and in three cases to 2.0 ng/ml medium. The female system seems preferable on these grounds alone.

As is perhaps to be expected with bioassays there is considerable between assay variation. In the female assay system, standard dose-response curves were calculated from the two lower doses of synthetic LH-RF in half of the assays; from the two higher doses in the remainder (Figures 3.7, 3.8). Similarly, examination of the mean LH and FSH responses in both the male and female systems (Tables 3.1, 3.3) to the same doses of synthetic LH-RF shows the considerable variation experienced. In the male system, LH responses are not significantly different from one another when the mean response at one dose is compared to the mean response at the next higher dose. For FSH, differences between responses to doses of 2.0 and 8.0 ng/ml medium and between responses to doses of 8.0 and 33.0 ng/ml medium are significant ($p < 0.05$, $p < 0.01$ respectively). In the female system differences between responses to doses of 1.9 and 7.5 ng/ml medium and between responses to 7.5 and 30.0 ng/ml medium are significant for both LH and FSH release ($p < 0.01$).

A further point of difference is that while FSH responses to similar doses of synthetic LH-RF are similar in both systems,

LH responses are markedly enhanced in the female system, such that comparison of LH responses to similar doses of synthetic LH-RF reveals that there is a 3 - 4 fold increase in LH responses in the female system when compared to the male system. There is no correlation between slopes of dose-response curves for LH and FSH in either the male or the female systems.

In terms of convenience and economy it is clear that the female assay system using the OOP rat has clear advantages, both in assay costs and in time required for dissection of pituitary glands.

The fact that of the various fragments of LH-RF tested for LH and FSH releasing activity only the N-terminal nonapeptide consistently exhibited such activity, is in keeping with the observation of a number of previous workers that the entire chain length and overall conformation of the decapeptide is essential for its activity (Schally, Arimura, Carter, Redding, Geiger, Konig, Wissman, Jaeger, Sandow, Yanaihara, Yanaihara, Hoshimoto and Sakagami, 1972; Burgus, Monahan, Rivier, Vale, Ling, Grant, Amoss & Guillemin, 1973; Fujimo, Kobayashi, Obayashi, Shinagawa, Fukuda, Kitada, Nakayama, Yamazaki, White & Rippel, 1972). The potency of the 1 - 9 nonapeptide in the present study was 6 - 13 % of that of the decapeptide; this is similar to that found by Burgus et al. (1973).

The absence of significant gonadotrophin release from pituitary glands incubated with pGLU-GLN-ALA-NH_2 and pGLU-TYR-ARG-TRP-NH_2 at the dosages used could be expected from the low activity of these peptides found by other workers (Schally et al., 1972). The greatly decreased biological activity of the peptide sequences tested when compared to that of the decapeptide could be due to spatial alteration or absence of functional groups required for postulated receptor binding, or to enhanced vulnerability to proteolysis, or indeed simply to intrinsic releasing activity unrelated to their structural similarities to the decapeptide. Investigation of the tertiary structure of LH-RF by Xray-crystallographic means has failed to provide any positive information about the arrangement of the molecule in space, other than that it appears to be disposed linearly (Dr. N. Isaacs, personal communication).

The synthetic peptides have been tested for in-vivo activity in constant oestrous rats (Gregory & Walpole, personal communication). While the decapeptide consistently caused ovulation at a dose of 0.25 µg i.v., the N-terminal nonapeptide failed to do so at a dosage of 50 µg i.v. The remainder of the peptides failed to cause ovulation at doses up to 5 mg i.v.

Other in-vitro assay techniques may prove to be superior to assays based on short term incubations of anterior pituitary glands. Pituitary cell culture techniques such as that described by

Vale, Grant, Amoss, Blackwell & Guillemin (1972) may provide sufficient numbers of homogeneous populations of responsive cells to overcome the prime objection to the sort of assay described herein, namely the heterogeneity of the assay tissue employed. Regrettably, cultured pituitary cells lose their responsiveness to both LH-RF and TSH-RF after 2 weeks of culture (Vale et al., 1972). Another promising technique is the production of pituitary cell suspensions, in which homogeneous populations of anterior pituitary cells are produced from fresh tissue by various enzymatic and mechanical breakdown steps (Hopkins & Farquhar, 1973). These cells remain viable in incubation medium for several days and respond readily to addition of synthetic LH-RF to medium by secretion of LH into the medium (Dr. C. Hopkins, personal communication).

Another form of assay is the pituitary superfusion technique of Serra & Midgley (1970). The great difficulty with this form of assay is that it is seldom possible to obtain a sufficient number of sequential responses from a single superfused pituitary for the calculation of adequate dose-response curves. Repetitive stimulation of superfused tissue may lead to exhaustion or decline in responsiveness of the tissue, although Smith and Follet (1972) found that with the quail anterior pituitary gland, superfusion for up to ten hours with many treatments results in the

release of only 20 % of the stored content of LH. A further problem is the risk that some treatments may have persistent effects on the superfused tissue which may interfere with the response to a subsequent treatment.

SECTION II IMMUNOASSAY

3.7. HISTORY

Hormones, especially peptide hormones, are present in plasma at concentrations as low as 10^{-12} to 10^{-10} M. Regrettably, they seldom contain elements that would allow their detection by specific chemical means, and are present in plasma and in other tissues in a milieu in which the total protein concentration may be as great as 10^{-4} to 10^{-3} M. Hence, for their detection and quantitative assay, highly specific and delicate methods are required. Immunological reaction between proteins has both great specificity and delicacy and has been used widely for the measurement of hormonal substances. While several immunological assay techniques have been developed, radioimmunoassay, (RIA), which is characterised by high degrees of sensitivity, specificity, precision and simplicity has been most widely used. The chief disadvantage of the method is that measurements made by RIA are not necessarily directly related to biological effectiveness. Hormonal potency must, by definition, be expressed in terms of some effect upon a biological system. Therefore, if feasible, it would be appropriate to utilise bioassay procedures for the measurement of plasma hormones.

The previous Section has demonstrated some of the difficulties encountered in the establishment of such bioassays. The advent of a RIA of a hormone is therefore an event of great importance, always

remembering that RIAs do not directly measure biological potency or effectiveness but rather measure the presence of a substance (or substances) possessed of a particular and often unique chemical structure.

RIA began with the observation of Berson, Yalow, Bauman, Rothschild & Newerly in 1956 that human subjects treated with pork and beef insulin developed globulin antibodies capable of binding ^{131}I-labelled insulin. Berson *et al.* (1956) showed that the percentage of labelled insulin bound to antibody decreased as the insulin concentration in the incubation mixtures was increased, and that unlabelled insulin could displace insulin from the insulin-antibody complexes.

The principle of competitive inhibition derived from these observations is summarised in the following reaction:

$$\underset{(F)}{(\text{free hormone})\text{-}\,{}^{131}I} + \begin{matrix}(\text{hormone}) \\ (\text{antibody}) \\ + \\ \text{unlabelled hormone} \\ \text{(in known standards} \\ \text{or body fluids)}\end{matrix} \rightleftharpoons \underset{(B)}{(\text{antibody bound})\text{-}\,{}^{131}I}$$

$$\Downarrow\Uparrow$$

antibody-bound unlabelled hormone

As a result of the competitive inhibition the ratio of the concentrations of antibody-bound (B) to free (F), or unbound labelled

hormone, denoted by $B/_F$, diminishes as the concentration of unlabelled hormone increases. The concentration in an unknown sample can be obtained by comparing the inhibition observed with that produced by standard solutions containing known amounts of hormone.

The above simple discussion is sufficient to use as a background for the discussion of the recent development of a RIA for LH-RF.

The requirements for a RIA are:

(1) The substance or a complexed form of the substance must be antigenic in some animal species, and cause the production of antibodies of a sufficiently high titre and specificity.

(2) The substance must be available in a highly purified form for labelling with a radioisotope to give a product with a suitable specific activity.

(3) The substance must be available in a suitable form for the standard.

(4) A method must be available to separate the free and bound antigen in the system.

Synthesis of LH-RF (Matsuo,Arimura, Nair & Schally, 1971) has made available the large amounts of this compound necessary for development of a RIA. Radioimmunoassay techniques for similar compounds, vasopressin (Miller & Moses, 1969), angiotensin (Catt,

Cain & Coghlan, 1967) and thyrotrophin releasing hormone (Bassiri & Utiger, 1972) have been reported recently.

The first report of a RIA for LH-RF was made by Kerdelhue and Jutisz (1972). They generated a specific antiserum in the guinea pig against a synthetic decapeptide (Hoffman - La Roche Laboratories, Basel, Switzerland). This antiserum, at a final dilution of 1:100 was able to bind 35 % of I^{131} labelled LH-RF. No cross reactivity was observed with oxytocin, lysine-vasopressin, synthetic thyrotrophin releasing factor (TSH-RF), ovine LH, FSH and prolactin. They employed their method for the detection of LH-RF in sheep median eminence stalk extract and plasma. Subsequently, Nett, Akbar, Niswender, Hedlund and White (1973), Jeffcoate, Fraser, Gunn & Holland (1973) and Arimura, Sato, Kumasaka, Worobet, Debeljuk, Dunn & Schally (1973) developed further radioimmunoassays.

The RIA used in the studies to be described was that of Nett et al. (1973). They obtained antiserum to the LH-RF analogue, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-COOH conjugated to the ε-amino groups of lysine residues in bovine serum albumin (BSA) by the mixed anhydride reaction (Niswender and Midgley, 1970), and estimated by incorporation of LH-RF-^{125}I that approximately 20 LH-RF-COOH molecules were conjugated to each BSA molecule. The LH-RF-BSA conjugate was emulsified in complete Freunds adjuvant and injected intradermally at multiple sites along the backs of rabbits (Vaitukatis,

Robbins, Neischlag & Ross, 1971). Booster immunisations with conjugate and incomplete Freunds adjuvant were administered at approximately monthly intervals, and the rabbits were bled by ear vein incision. A usable antibody titre to the antigen was obtained within three months.

The RIA developed by Jeffcoate et al. (1973) similarly used antiserum raised in rabbits. Two ng of synthetic decapeptide were conjugated to 2 ng BSA using 75 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 0.25 ml water (Goodfriend, Levine & Fasman, 1964). After dialysis overnight, 1 mg of the conjugate in 2 ml water, emulsified in Freund's complete adjuvant, was injected into 20 intradermal sites in a white New Zealand rabbit. A blood sample was obtained eight weeks after this primary immunisation and the assay developed using this antiserum.

The RIA of Arimura, Sato, Kumasako, Worobec, Debeljuk, Dunn & Schally (1973) also used antiserum developed in rabbits, although they employed synthetic LH-RF adsorbed to polyvinyl-pyrrolidone as an antigen. They report two types of RIA, a conventional type, in which addition of unlabelled hormone in quantities greater than 0.31 ng/tube led to competitive inhibition of binding of labelled LH-RF, and a so-called "paradoxical binding" assay, in which the addition of small amounts (less than 0.31 ng/tube) of unlabelled LH-RF actually enhanced binding of labelled LH-RF.

The RIA of Nett et al. (1973) was carefully examined by them with respect to its specificity, and its capacity to measure LH-RF in material from a variety of animals. In their system, when 200 µl of the anti-LH-RF serum was used at a dilution of 1:800 approximately 40 % of the LH-RF-^{125}I was bound to antibody in the tubes containing no competing unlabelled hormone. Inhibition curves showing the decreased amount of LH-RF-^{125}I bound by antibody due to increasing levels of serum from castrate sheep, and of crude hypothalamic extracts of porcine, ovine and Rhesus monkey origin, were parallel to those obtained with synthetic LH-RF in the region where the quantity of LH-RF-^{125}I bound to antibody was equal to 20 to 80 % of that bound in the buffer control tubes.

The sensitivity of the assay as reported by Nett et al. (1973) i.e. the least amount of LH-RF which resulted in significantly less binding of LH-RF-^{125}I than that in the tubes containing no competing unlabelled hormone, ranged from 1.0 to 3.8 pg in seven different assays. Exogenous LH-RF was quantitatively recovered when varying amounts, added to 200 µl of serum from a hypophysectomised ewe, were assayed.

Specificity of the anti-LH-RF-serum was tested by determining the ability of various analogues of LH-RF to inhibit the binding of LH-RF-^{125}I to antibody. A number of analogues in which the terminal amino group of LH-RF was replaced by other groups or amino acids

(e.g. -COOH, -O-CH_3, -NH-CH_3, -Tyr, -β-Ala-NH_2) demonstrated relative activities of 0.7 to 1.0. Any greater manipulation of LH-RF such as shortening the chain or replacing amino acids within the chain with other amino acids produced relative activities of less than 0.2. Neither thyrotrophin releasing factor, nor melanocyte-stimulating hormone inhibiting factor exhibited any cross reactivity.

A comparison was also made with a bioassay system (White, Hedlund, Rippel, Arnold & Fluoret, 1973) after porcine stalk median eminence extract had been subjected to column chromatography on Sephadex G-25. Relative potencies of successive fractions by bioassay and by RIA were in close agreement, and the peak of activity was found in the same fraction by both assay systems.

3.8. Experimental Methods

A. Radioimmunoassay

Radio-iodination of LH-RF

Synthetic LH-RF (I.C.I.; Macclesfield) was radioiodinated according to the method of Niswender, Reichert, Midgeley and Nalbandov (1969) with some modifications.

2.5 µg of LH-RF was radioiodinated with 1 mCi of ^{125}I (Code IMS.30, Radiochemical Centre; Amersham) for 15 sec. in the presence of 20 µg chloramine T(BDH; Poole); in a small plastic phial, the

reaction being stopped by addition of 40 µg of sodium metabisulphite (BDH; Poole). LH-RF-^{125}I and free ^{125}I were separated on a column (0.75 x 20 cm) of Sephadex G-25 (Pharmacia; Sweden). The column was prepared and eluted with 0.01 M phosphate buffered saline containing 0.1 % gelatin (gel-PBS). The free ^{125}I eluted in 10 to 13 ml whereas LH-RF^{-125}I eluted in 13 to 17 ml. Successive 1 ml eluate volumes were counted on a well type scintillation counter and automatic scaler (Ekco Electronics; London). A typical elution profile appears in Fig. 3.12. Tubes containing radioiodinated LH-RF were stored at -15^{o}C behind lead shielding until used.

Radioimmunoassay

Radioimmunoassays were carried out using a protocol similar to that previously reported for steroid hormones by Niswender and Midgley (1970). Assays were carried out in polystyrene tubes (LP3, Luckhams Ltd.; Sussex), capacity 2 ml. Actual contents of tubes are displayed in table form in Table 3.7. Wherever possible some samples (pools) were assayed in several successive assays to provide estimates of inter-assay variation. Reagents are listed in Appendix 3.4.

Day I 50 - 100 µl of plasma (or other material to be assayed) diluted to 400 µl with gel-PBS, 200 µl of anti-LH-RF serum at dilutions of 1:80,000 or 1:100,000 in gel-PBS-EDTA-NRS, and 200 µl of LH-RF-^{125}I were added to assay tubes. The amount of LH-RF-^{125}I added

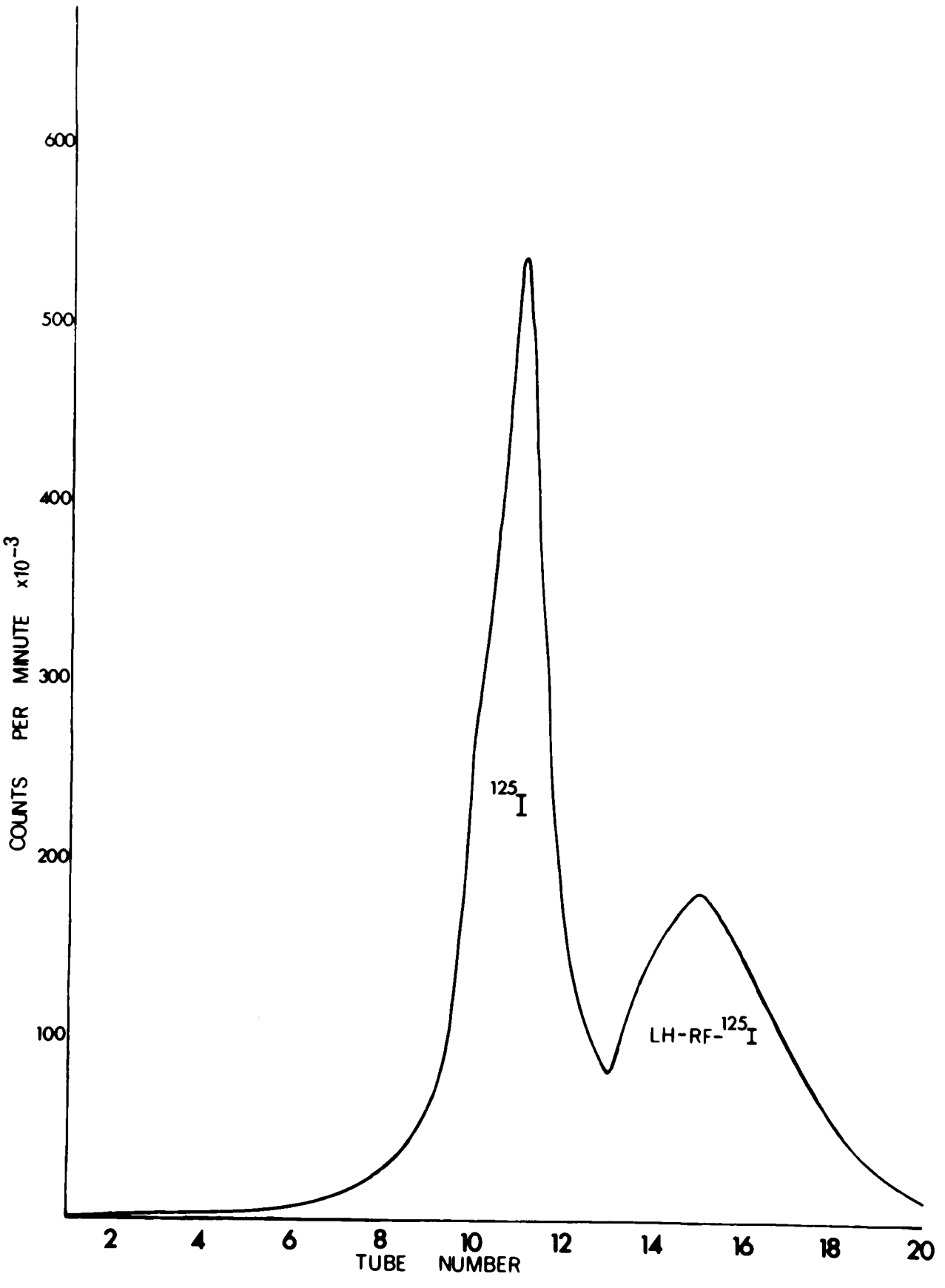


Figure 3.12 Elution profile of ^{125}I and LH-RF-^{125}I on Sephadex G-25.

	DAY I				DAY II	DAY III
REAGENT	BUFFER	SAMPLE OR STANDARD	ANTISERUM	LH-RF ^{125}I	ANTI GAMMA GLOBULIN	PBS
TUBES 1, 2, 3 (TOTAL COUNT)	-	-	-	200 µl	-	-
TUBES 4, 5, 6 (BLANK)	400 µl	-	200 µl (buffer without antiserum)	200 µl	200 µl	1.0 ml
TUBES 7, 8, 9, 10 (TOTAL BOUND)	400 µl	-	200 µl	200 µl	200 µl	1.0 ml
STANDARDS	200 µl	200 µl	200 µl	200 µl	200 µl	1.0 ml
POOLS &	300 - 350 µl	50 - 100 µl	200 µl	200 µl	200 µl	1.0 ml
SAMPLES	200 - 375 µl	25 - 200 µl	200 µl	200 µl	200 µl	1.0 ml

TABLE 3.7 Chart of constituents in various assay tube groups.

varied from 20 to 70 pg depending on the specific activity (300-1000 μCi/μg) of the particular radioiodinated LH-RF used. Duplicate standard curves with 9 points ranging from 6 pg to 1 ng were included in each assay, while plasma samples were assayed in duplicate wherever possible. Tubes were mixed (Whirlimixer, Fisons; Loughborough) for 1 second and incubated in a cold room at 4°C for 24 hours.

Day II 200 μl of anti-rabbit gamma globulin precipitating serum (Wellcome; Beckenham) diluted to 1:30 with PBS were added to tubes which were again mixed and incubated as above.

Day III 1 ml of PBS at 4°C was added to tubes after which the tubes were centrifuged at 3000 r.p.m. for 30 minutes (Mistral, 6L, MSE; London), the supernatant removed by aspiration, and the precipitates were counted in an automatic gamma counting system (Series 1185, Nuclear Chicago, Illinois).

Operations involving addition of reagents etc. to tubes were carried out in ice filled trays so that tubes were maintained at all times at 0 - 4°C.

Dose-Response Curves

The ratio of bound (B) to free (F) tracer versus bound tracer is referred to as a Scatchard plot (Scatchard, 1949). However, the

plot of B/F versus B is inconvenient for the estimation of potency. It is more convenient to plot the ratio of bound tracer (B) to the number of counts precipitated in the absence of an unknown or standard (Bo) versus the log dose of standard X. This standardises the scale (from 0 - 100 %) and obviates the need to calculate B/F. Moreover, since the error in the denominator is small and uniform, the B/Bo percentage is more stable than the B/F ratio (Rodbard, Rayford and Ross, 1970). The plot of B/Bo versus log X results in a sigmoidal curve, which can be approximated by a linear segment for at least a tenfold range of doses, thereby facilitating potency estimation. This form of the dose response curve is non-linear. An example of such a curve can be found in Figure 3.13.

When the logit transformation, logit B/Bo = $\log_e \left[\frac{B/Bo}{1 - B/Bo}\right]$ is applied to B/Bo, the sigmoidal curve just described becomes almost linear; see Figure 3.14. The curve does not depart significantly from linearity between 10 and 90 % or for logits between -2.2 and +2.2. Accurate estimation of slope and intercept, either by means of linear-regression analysis or graphical methods, is thereby permitted.

Quality Control

Several parameters describing the assay have been selected and appear in Table 3.8. and are plotted graphically in Figure 3.15. No record of specific activity of tracer, or of amount of tracer per

Figure 3.13 Plot of $B/_{Bo}$ % versus log dose of LH-RF in RIA for LH-RF.

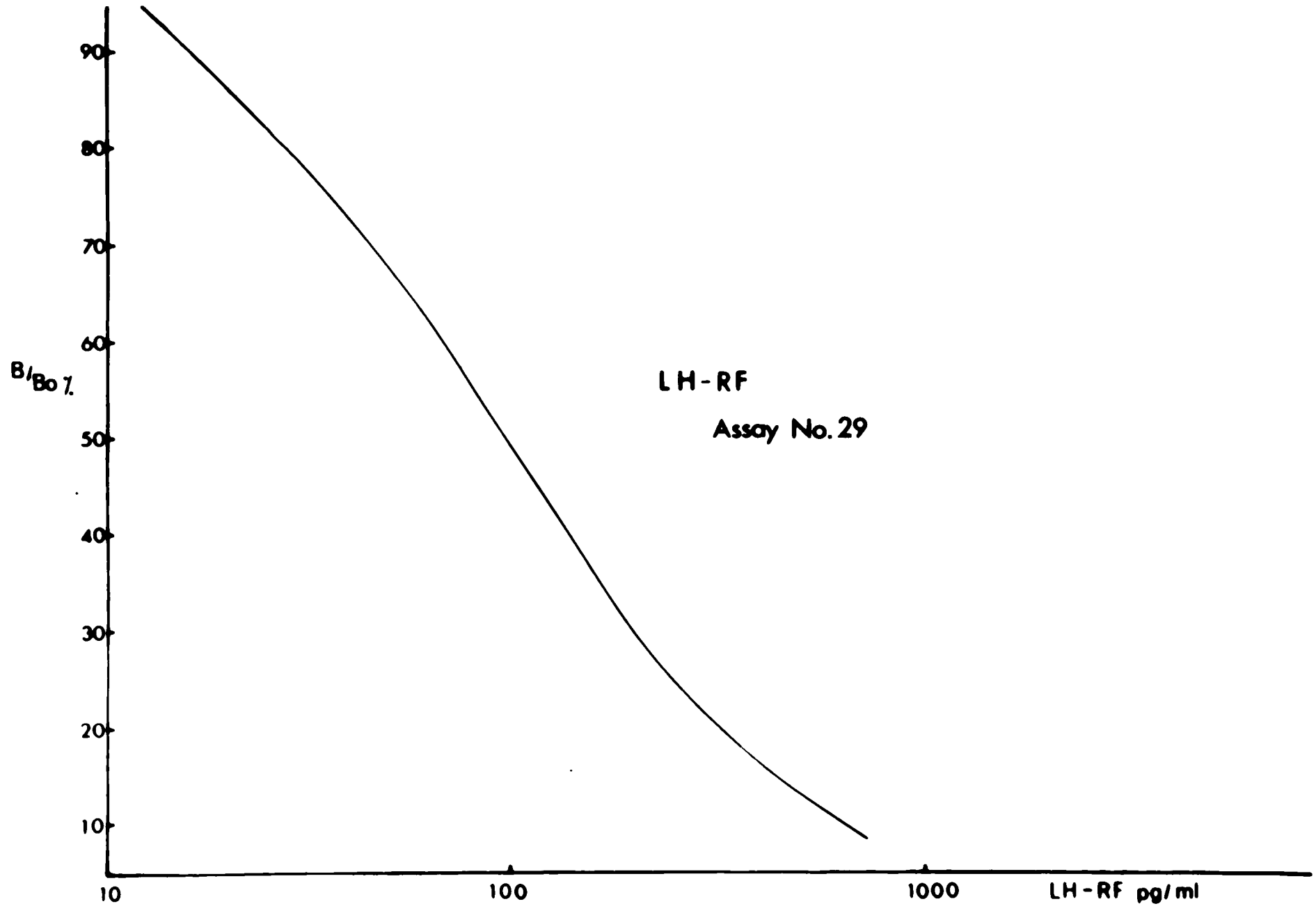


Figure 3.14 Plot of logit $B/_{Bo}$ versus log dose of LH-RF in RIA for LH-RF.

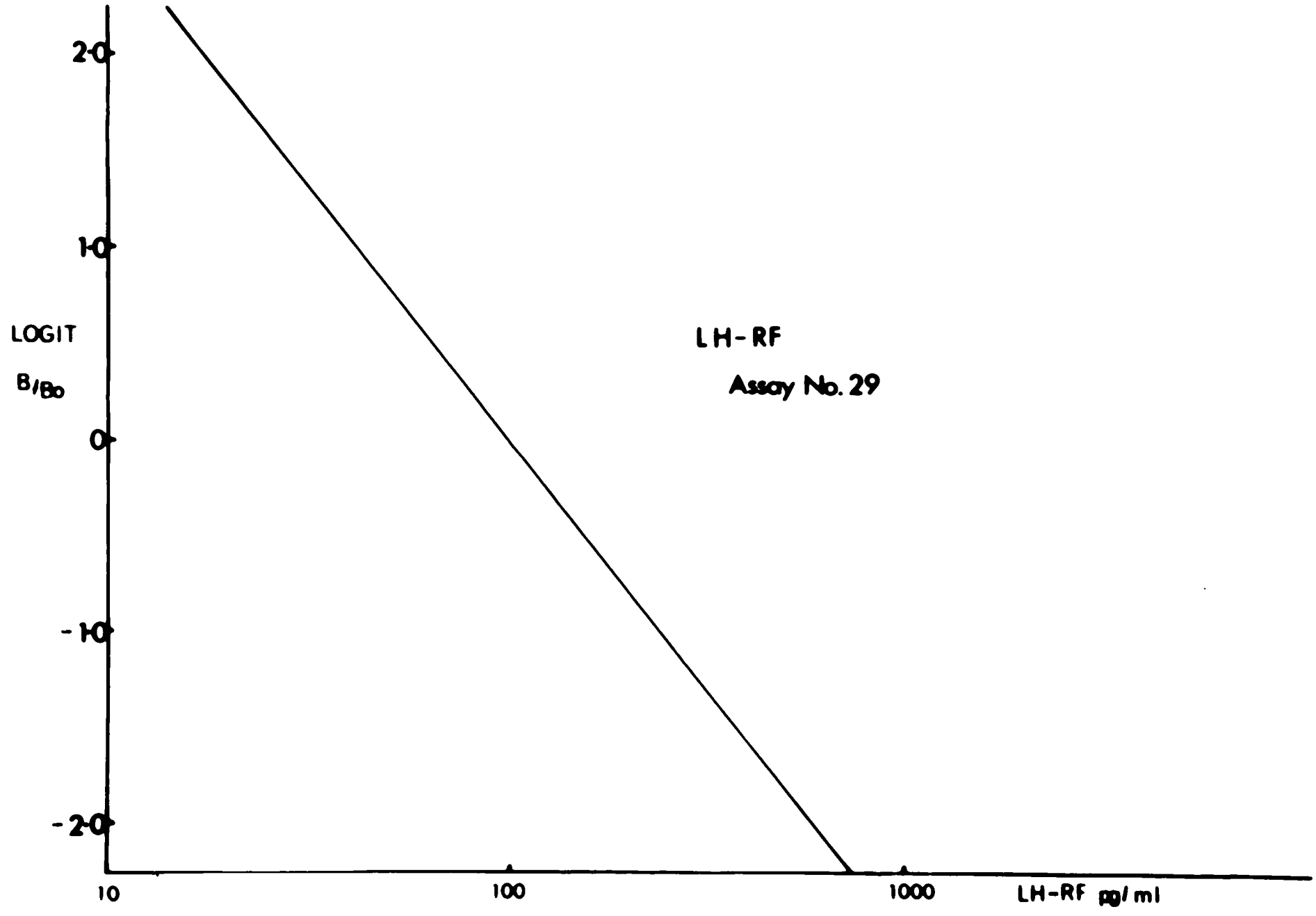
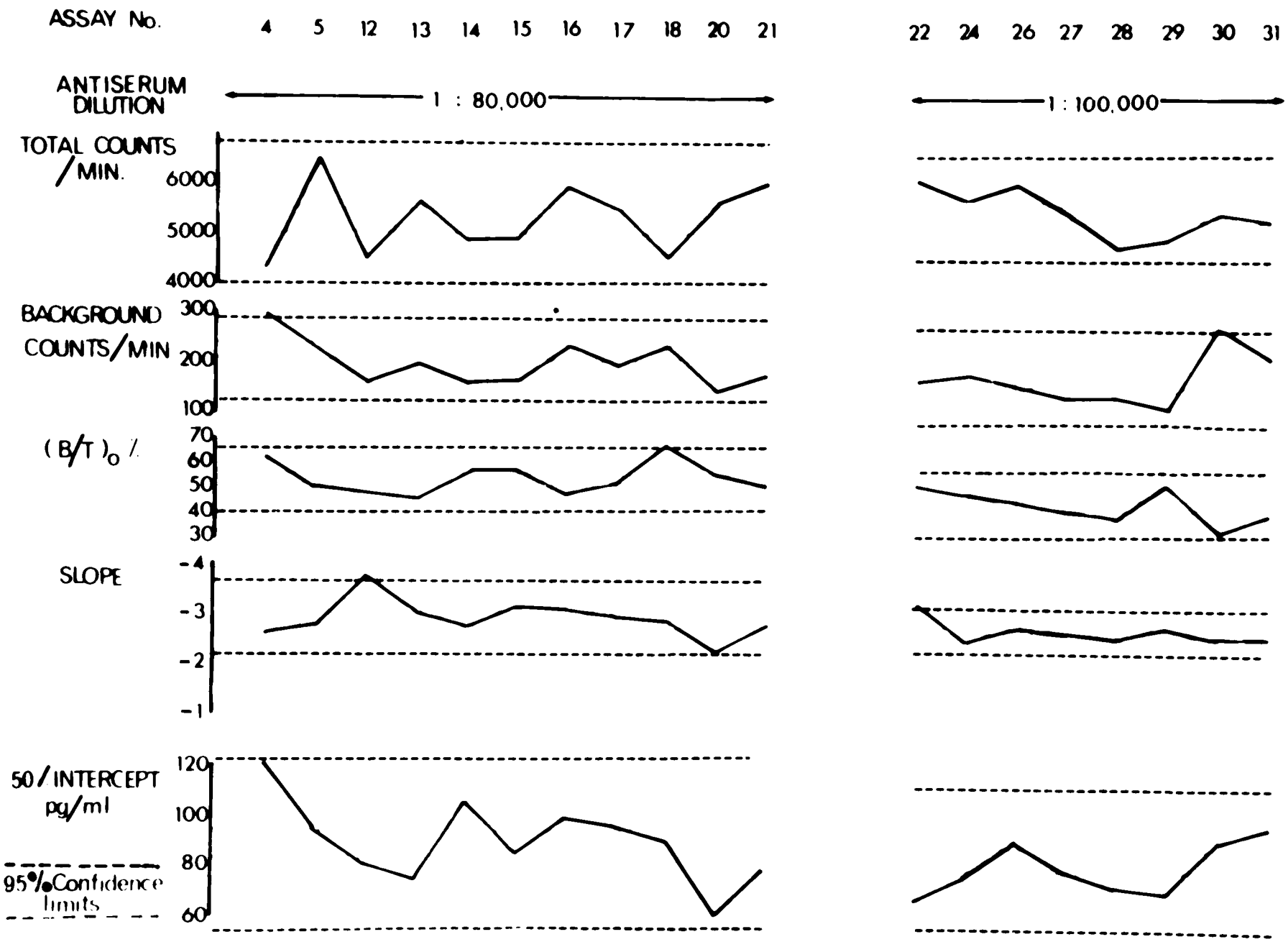


TABLE 3.8

ASSAY NO	TOTAL COUNTS /MIN	BACKGROUND COUNTS/MIN	(B/T)o %	SLOPE	50 % INTERCEPT pg/ml
	ANTISERUM	DILUTION	1:80,000		
4	4345	292	61.1	-2.57	120
5	6524	221	49.5	-2.77	94
12	4501	158	47.5	-3.74	80
13	5617	198	45.6	-2.97	67
14	4901	155	55.0	-2.73	105
15	4976	162	54.6	-3.07	84
16	5936	228	47.6	-3.03	98
17	5538	191	51.6	-2.96	95
18	4054	232	66.1	-2.83	90
20	5672	139	55.1	-2.19	60
21	6002	167	51.2	-2.69	78
MEAN ± S.E.M.	5278 ± 235	214 ± 14	53.2 ± 1.9	-2.87 ± 0.11	58 ± 5
	ANTISERUM	DILUTION	1:100,000		
22	6102	163	50.6	-3.18	67
24	5794	173	47.2	-2.48	76
26	6070	154	44.5	-2.68	90
27	5540	128	41.1	-2.04	78
28	4731	132	38.2	-2.52	72
29	4872	108	52.2	-2.73	70
30	5422	269	32.6	-2.58	100
31	5344	201	39.9	-2.50	106
MEAN ± S.E.M.	5484 ± 179	166 ± 17	43.3 ± 2.3	-2.66 ± 0.08	82 ± 5

TABLE 3.8. Quality Control Data for LH-RF radioimmunoassays. Assay Nos. missing in sequence refer to check assays, or to assays using other antisera in which all the above data are not available.

Figure 3.15 Quality Control Parameters in RIA for LH-RF (see text).



assay tube appears. These parameters are not independent of total counts in each assay, and have not been recorded primarily because of difficulties in counting samples with very high gamma emissions.

Total counts, T. The "Total Counts" applied to each assay tube is proportional to the counting efficiency of the detector and to the amount of tracer present divided by the specific activity. The number of counts determines the counting error and indirectly influences the counting errors for Bo, B and N (the number of counts that are non-specifically precipitated, or "trapped"). If T becomes too low, then the overall error in B/T increases rapidly. T has been maintained between 4000 and 6000 cpm, and determined in triplicate.

Nonspecific, or background counts, N. A value greater than 5 % of the total counts is indicative of trouble in RIA systems (Rodbard et al., 1970). N may be affected by the incubation conditions and by the efficiency of the separation technique. N has been determined in triplicate, "Blank" tubes and in two of 19 assays has proved greater than 5 % T.

(B/T)o. This is the total bound counts (minus N) divided by the total counts T (minus N), when only tracer is present. In the present assay, this quantity, the 100 % control, is obtained from four "Total Bound" tubes containing antibody and labelled tracer but with no added unlabelled hormone. The (B/T)o is an important parameter describing

the performance of an assay. It may be low for a variety of reasons, low binding affinity or titre of antibodies, incomplete separation of bound from free hormone or failure to reach equilibrium. Whenever (B/T)o is low the assay precision will be decreased. In the present assay, (B/T)o varies from 45.6 % to 66.1 % (53.2 $\pm$ 1.9) when the antiserum dilution was 1:80,000 (n = 11) and from 32.6 % to 52.2 % (43.3 $\pm$ 2.3) when the antiserum dilution was 1:100,000 (n = 8). Although the dilution of antiserum which provides for maximum precision differs somewhat from the dilution which provides for maximum sensitivity, values of (B/T)o in the region of 40 - 60 % provide an adequate compromise.

Slope. The slope of the dose-response curve is constant when an assay is "under control". The slopes have been determined graphically by plotting the curve on logit-log paper (see Figure 3.14). Rodbard et al. (1970) have found that the graphically determined slope is virtually identical with that calculated by linear-regression methods. The standard error of the slope provides little additional information, since the heteroscedasticity of the data vitiates its applicability. In the present RIA system the slopes for assays using antiserum diluted at 1:80,000 range from -2.19 to -3.74 (-2.87 $\pm$ 0.11) and for assays using antiserum diluted at 1:100,000 range from -2.48 to -3.18 (-2.66 $\pm$ 0.08).

Abscissa intercept. Estimates of the sensitivity of assays can be

made by comparing minimal detectable concentrations of hormone between different assays. Since the dose response curve is rarely reliable for interpolation above the 90 % response (because of loss of linearity) the 90 % intercept may be used as one such measure. However, the 90 % response is rather variable and here the 50 % intercept (50 % I) on the x axis is preferred. The 50 % I for assays with antiserum diluted at 1:80,000 range from 60 - 120 (88 $\pm$ 5) pg and for assays with antiserum diluted at 1:100,000 from 67 - 106 (82 $\pm$ 5)pg.

Within-assay and between-assay variability. These parameters can be assessed by including a small number of samples in duplicate within each assay, and repeating estimations of these same samples in successive assays. Because of the relatively low concentrations of LH-RF to be found in peripheral plasma it has proved difficult to establish pool sample estimations in more than a few successive assays amongst those listed in Table 3.8. For example in Assays 14, 15, and 16, pool samples consisted of human plasma obtained five minutes after intravenous injection of 50 µg LH-RF, and in those assays gave concentrations of 3150, 2800, and 2700 pg/ml respectively, when estimated in duplicate. In assays subsequent to Assay 30 two pool samples have been included, one consisting of normal male rat serum containing added synthetic LH-RF such that the concentration was 250 pg/ml, the other consisting of normal male rat serum containing an acid extract of a single normal male median eminence such that the

concentration was 250 pg/ml.

The lack of adequate pool samples in this relatively early stage of development of the assay means that assessment of whether the assay is "in control", depends on the (B/T)o and the 50 % intercept. These four parameters are thought by Rodbard et al. (1970) to be the most informative parameters for such assessments. In reference to Figure 3.15 it can be seen that while the (B/T)o parameter is reasonably stable, there is considerable variation in the 50 % Intercept. It becomes of great importance in these circumstances that comparisons of physiological interest be made on the same assay. In the result section of this and the subsequent chapter, occasions on which comparisons are made between samples assayed in different assays are noted.

Specificity of LH-RF RIA

The synthetic fragments of LH-RF previously discussed in Section 3.5. were included in an assay at dilutions ranging from 15 to 1000 pg/ml. None of the fragments was capable of inhibiting binding of tracer to antiserum. These results, added to those of Nett et al. (1973) noted in Section 3.8 suggest that this RIA is specific for the decapeptide LH-RF.

Interference by plasma with binding in RIA.

In developing a new assay, it is important to ensure its

validity as a measuring system by demonstrating that endogenous plasma hormone and standard hormone react identically with the antibodies, and that conditions are maintained such that other substances in plasma do not affect the determination. A necessary, though not sufficient condition for identical reactivities of standard and unknown is that the concentration calculated for undiluted plasma must be the same, regardless of the dilution of plasma used for assay. It has been observed, however, that serum or plasma, quite apart from their hormone content have a small effect on RIA systems (Odell, Rayford & Ross, 1967; Burr, Grant, Sizonenko, Kaplan & Grumbach, 1969), binding to antiserum often being slightly depressed. In the present assay, it has been found that with increasing volumes of plasma in assay tubes (particularly above 100 µl), the rise in hormone concentration tends to be non-linear. Examples of this problem appear in Table 3.9 in which the results of determinations of LH-RF concentrations in different aliquot volumes of the same samples appear. Characteristically, determinations of LH-RF concentration in 200 µl aliquots give rather lower results than might be expected from the results of determinations using 100 µl aliquots, particularly when LH-RF concentrations are low. When LH-RF concentrations were high, there was little difference in final LH-RF concentrations when assay tubes contained 25 - 100 µl of plasma. The specific plasma constituents contributing to this effect

	SAMPLE NO	Plasma in incubation mixture µl/ml				
		200	100	75	50	25
	1	33	48			
	2	36	53			
	3	24	46			
LH-RF	4	56	63			
pg/ml	5	43	65			
	6	-	124	128	127	136
	7	-	91	97	101	106
	8	-	-	292	298	280

TABLE 3.9. Effect on final calculated concentration of LH-RF in samples assayed using different volumes of plasma in assay tubes.

have not been investigated. Measurements, particularly those made for comparisons of physiological interest have been mostly made in equal plasma volumes of 100 µl or less. No plasma fillers have been used.

B. Operations on Animals

Extraction of Median Eminence

Male animals, 250 - 350 g were maintained as described in Chapter 2. Under urethane anaesthesia, (10 % solution, 10 ml/kg i.p.) animals were decapitated, and the ventral surface of the brain exposed by blunt dissection. After division of the pituitary stalk at its junction with the gland, the area of the median eminence was excised with fine scissors, the approximate dimensions of the tissue excised being 3 mm anteroposteriorly from the optic chiasma to the mammillary bodies, 4 mm laterally, and 1.5 mm supero inferiorly.

The tissue was immediately placed in 1 ml of 0.1 N HCl and homogenised for 2 minutes with a motorised homogeniser. The homogenate was centrifuged at 3000 r.p.m. at 4°C (Mistral, MSE) and the supernatant stored frozen. Prior to use, the extract was neutralised to pH 7.0 with 2 N NaOH.

Selection of male animals for copulation experiments

Virgin male animals, 300 - 350 g were maintained as described

in Chapter 2, except that they were kept under conditions of reversed light, lights on at 20.00, lights off at 10.00 h.

Animals were assessed with respect to mating behaviour by placing each male with a mature female injected s.c. 72 hours prior to test with 100 µg oestradiol benzoate.

Male animals were accepted for further experimentation if ejaculation (assessed both by observation of a copulatory plug in the vaginal introitus of the female and by observation of the characteristic post-copulatory upright stance and period of disinterest of the male) occurred within 15 minutes of introduction to the female. Only one such test of mating behavioural competence was made with each male.

Intra-cardiac catheterisation

Permanent intracardiac cannulae were inserted into male animals as described above according to the method of Greig (1971), with minor modifications.

Under tribromoethanol (Avertin, Winthrop; Surrey) anaesthesia, 10 ml/kg of a 2 % solution in N-saline, anteroposterior incisions were made at the back of the neck, and over the right external jugular vein. The cannula, inserted into the jugular vein, with the intravenous part ending in the right atrium, and the other end running subcutaneously

emerges at the back of the neck. The intravenous part of the cannula is composed of silastic rubber (0.02 in. i.d., 0.037 in. e.d.; Dow Corning Corp.; Michigan), and is joined by a metal junction (22-gauge hypodermic needle tubing) to Tygon tubing (0.01 in. i.d., thick walled; Technicon Instrument Corp.; New York). Cannulae were kept full of N-saline containing 100 IU/ml of heparin (Pularin, Evans Medical; Liverpool), and closed with stainless steel plugs, 0.025 in. in diameter. Cannulae were flushed daily with 0.10 ml of N-saline.

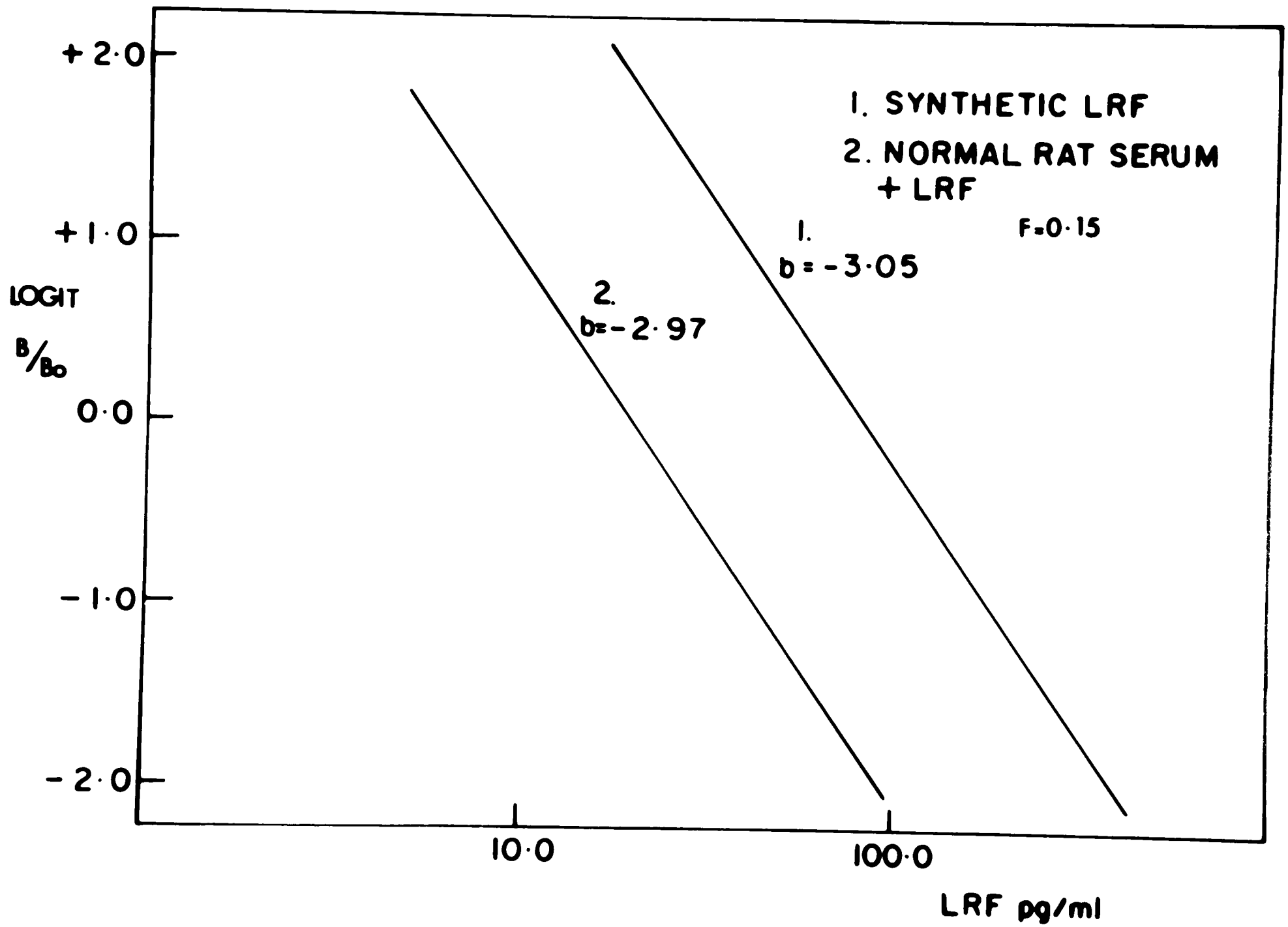
By connection of the cannula to a 1 ml syringe, blood can be withdrawn in conscious unanaesthetixed animals. Over a period of two weeks, approximately 10 % of cannulae fail.

3.9. RESULTS

It is essential to demonstrate in assay systems that successive dilutions of materials containing LH-RF, which are to be assayed, exhibit inhibition curves which are parallel to the inhibition curve produced by successive dilutions of the standard material. A number of such investigations have been carried out.

Normal rat serum with added LH-RF. Serum taken from normal male rats was mixed with synthetic LH-RF and assayed in 7 dilutions ranging from neat serum to a 1:64 dilution. The resulting inhibition-curves for the standard preparation and serum containing LH-RF appear in Figure 3.16.

Figure 3.16 Inhibition curves for LH-RF standard and for normal rat serum containing added synthetic LH-RF.



The dose-response curves calculated by linear regression ~~are parallel~~ ($F < 1.0$), do not deviate significantly from parallelism

Median Eminence Extract. Extract of the median eminences of two normal male rats were assayed in 7 dilutions ranging from neat extract to a 1:640 dilution. The inhibition curves appear in Figure 3.17. The dose-response curves ~~are parallel~~ ($F < 1.0$), do not deviate significantly from parallelism.

Portal plasma. Plasma from blood collected from the cut ends of the pituitary stalks of male rats during stimulation of the medial preoptic area (for details see Chapter 4) was assayed in five dilutions ranging from neat plasma to a 1:8 dilution. The inhibition curves for male plasma appear in Figure 3.18, the dose response curves being parallel ($F < 1.0$). Portal plasma, collected during medial preoptic area stimulation of female rats at various stages of the oestrous cycle (for details see Chapter 4) was assayed in as many dilutions as individual samples allowed. The dose-response curves as calculated for each individual sample were combined to give a "mean" dose response curve which appears in Figure 3.19. The slopes of the dose-response curves are sufficiently similar for measurements of LH-RF in female portal plasma collected during electrical stimulation of the medial preoptic area to be made with confidence.

Human female plasma containing LH-RF. Plasma taken from a female patient five minutes after the intravenous injection of 50 μg LH-RF

Figure 3.17 Inhibition curves for LH-RF standard and for rat median eminence extract.

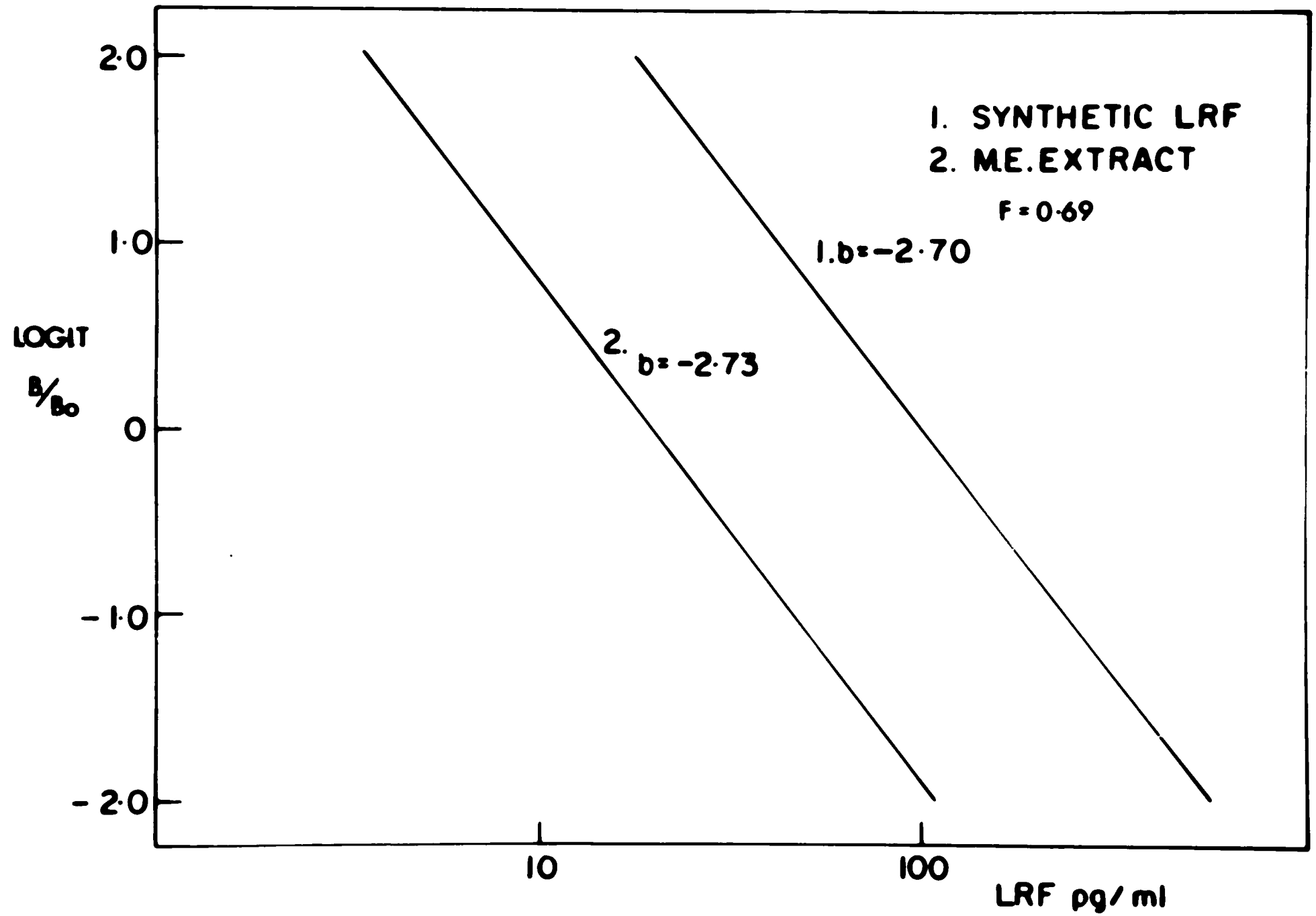


Figure 3.18 Inhibition curves for LH-RF standard and for portal plasma collected from male animals.

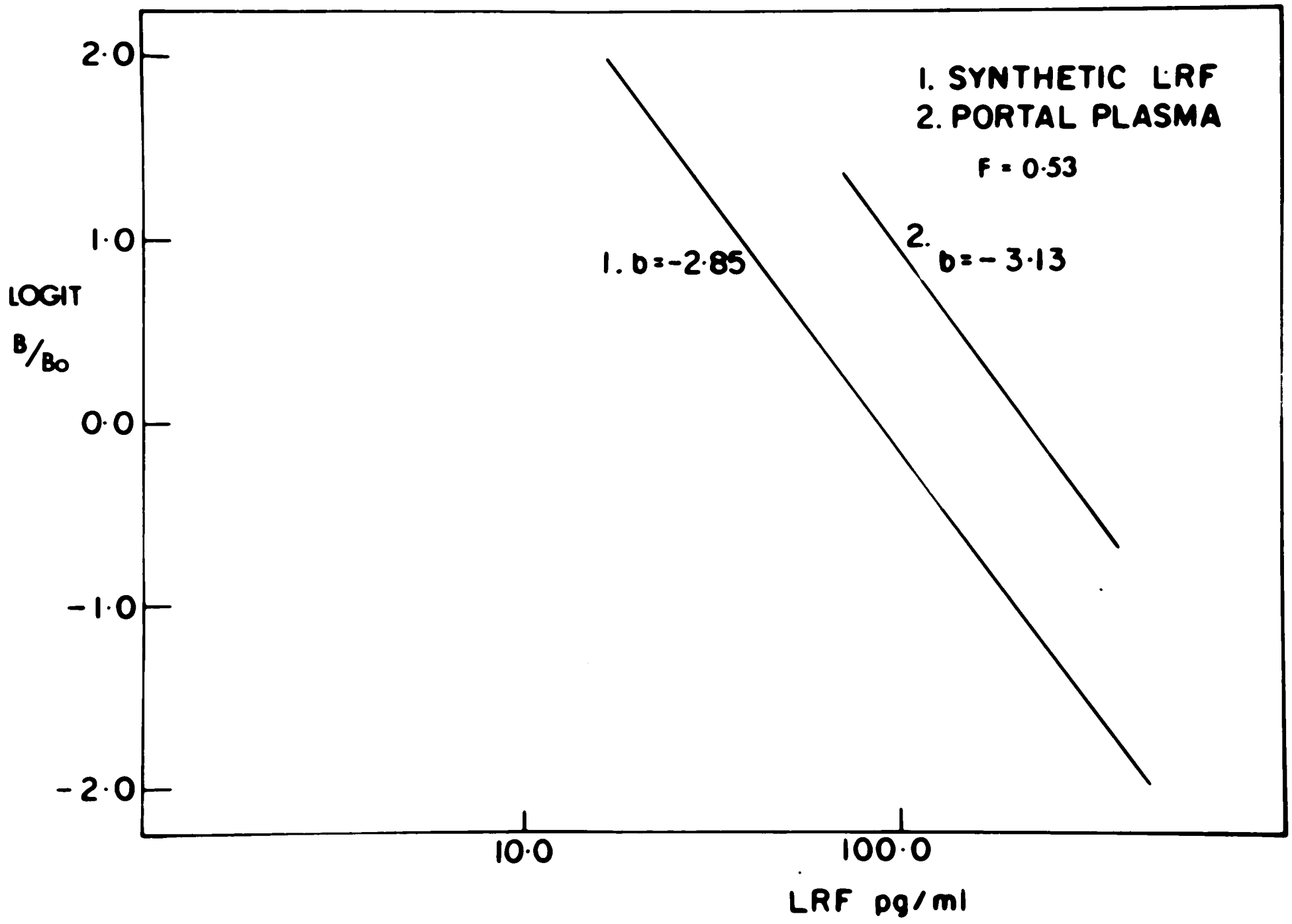
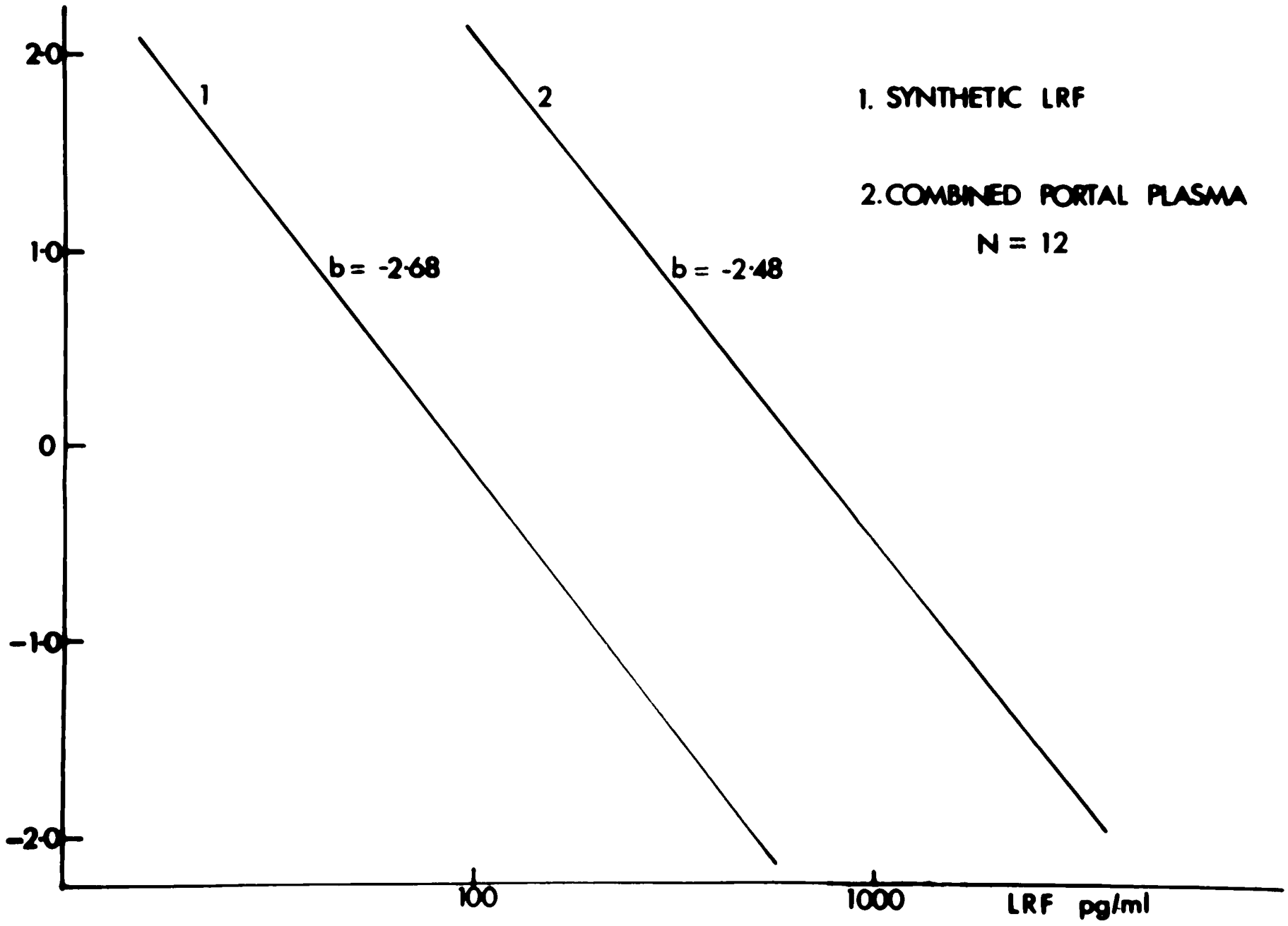


Figure 3.19 Inhibition curve for LH-RF standard and "mean" inhibition curve for samples of portal plasma collected from 12 female rats.



was assayed in seven dilutions ranging from neat plasma to a 1:1280 dilution. The dose-response curves appear in Figure 3.20 ~~and are parallel~~ ($F < 1.0$), and do not deviate significantly from parallelism.

Normal rat serum after intravenous injection of synthetic LH-RF and median eminence extract. In order to test whether synthetic LH-RF and median eminence extract could be measured after addition to plasma in-vivo, either 100 ng LH-RF or the extract of two normal male median eminences were injected intravenously in a volume of 0.5 ml into 200 g male rats anaesthetised with urethane which had received 2500 IU of heparin intravenously. Three minutes after injection, blood was withdrawn from the external jugular vein and assayed in serial dilutions. The resulting dose-response curves appear in Figure 3.21. The slopes of the curves are sufficiently similar for measurements of LH-RF in peripheral plasma to be made with confidence. Similar evidence of parallelism was found in six other animals treated either with synthetic LH-RF or median eminence extract.

Presence of LH-RF in plasma from post-menopausal women treated with oestrogen.

In experiments carried out with Dr. J. Bonnar of the John Radcliffe Infirmary, Oxford, concentrations of LH-RF in peripheral venous plasma were determined at intervals in fifteen women treated

Figure 3.20 Inhibition curves for LH-RF standard and for human serum containing synthetic LH-RF.

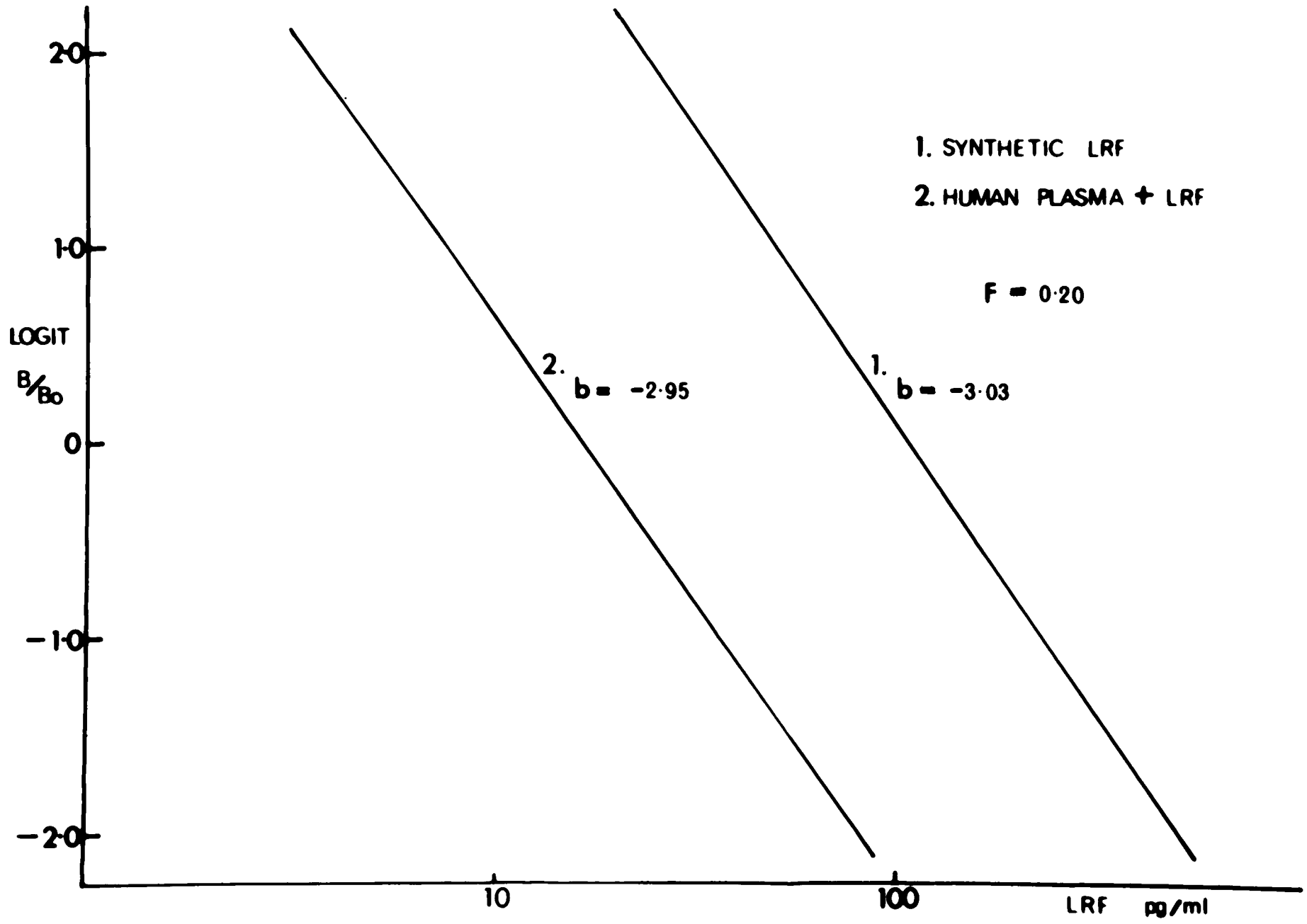
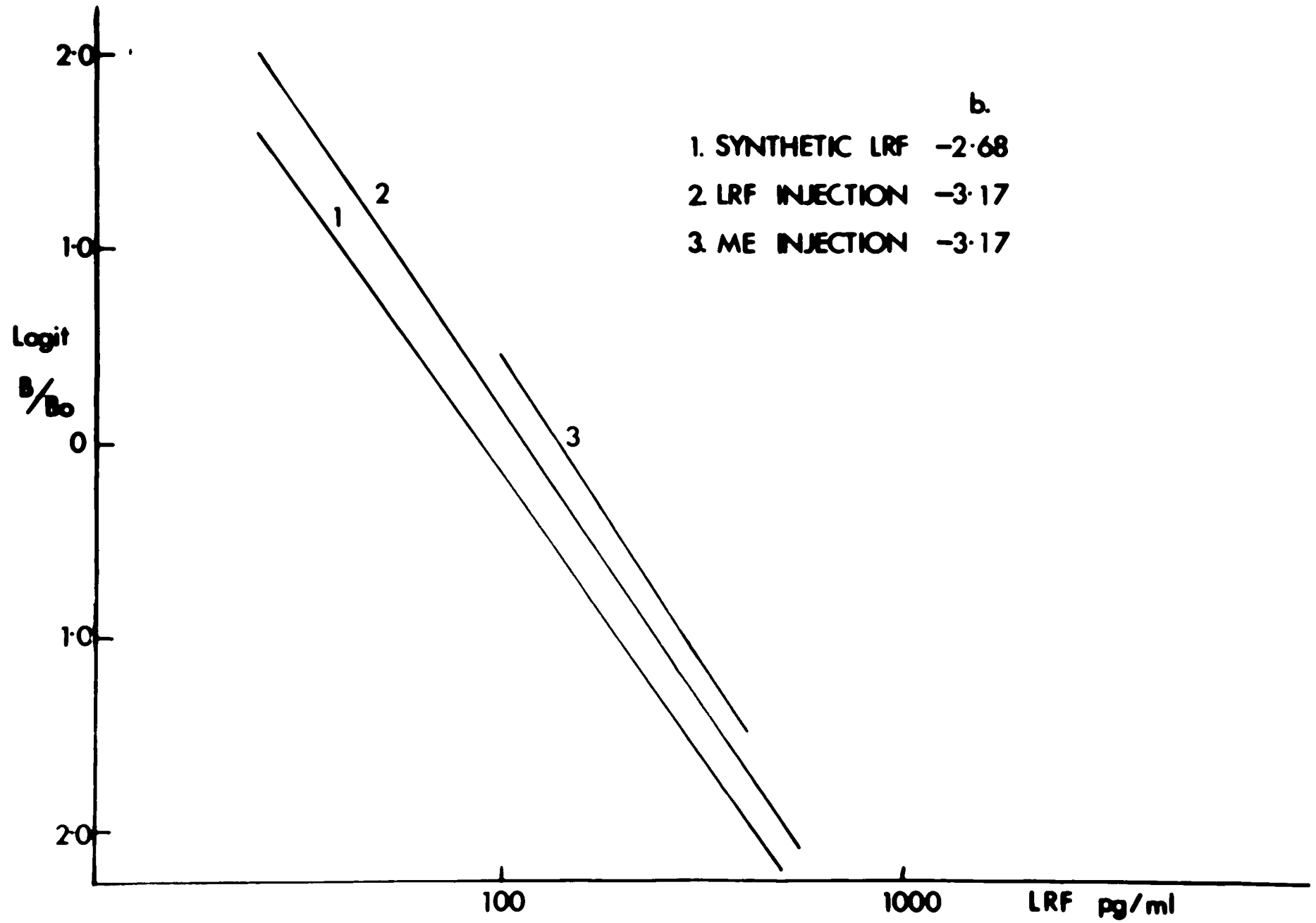


Figure 3.21 Inhibition curves for LH-RF standard and for rat serum from two animals; one injected i.v. with synthetic LH-RF, the other with median eminence extract.



with a subcutaneous implant of 25 mg. oestradiol benzoate for the relief of post-menopausal symptoms. A sample of blood was taken prior to implantation, and at 1, 2, 4 and 6 months after insertion of the implant. All samples were assayed in one assay in duplicate 200 µl aliquots. There was no evidence of any change in peripheral LH-RF concentration during treatment, the mean (± S.E.M.) concentrations of LH-RF in peripheral plasma prior to insertion of implant and at 1, 2, 4 and 6 months being 15.0 ± 0.8, 15.2 ± 0.5, 15.9 ± 1.4, 15.4 ± 1.0 and 16.3 ± 0.7 respectively.

Effects of sexual stimulation on peripheral LH-RF concentrations in male rats.

Male rats, selected and cannulated as described were introduced to mature female rats treated 72 hours previously with 100 µg of oestradiol benzoate. The time between introduction and ejaculation and the LH-RF concentrations of plasma samples taken prior to introduction, and at various times after ejaculation appear in Table 3.10, LH-RF measurements being made on single 200 µl samples. On occasions two experiments were performed with the same male animal at an interval of five days.

Generally, no change can be seen in plasma LH-RF concentration over the time period of sampling. The measurements in experiments 1, 2 and 3 were made in Assay No. 12, in which the 50 % intercept was

ANIMAL NO	TIME TO EJACULATION MIN	LH-RF pg/ml					
		Sample Time					
		Pre	0	2	5	10	20
1	10	*ND	19.4	22.3	21.6	24.0	ND
2a	5	54.0	47.0	47.0	30.5	ND	28.5
b	13	ND	29.5	42.0	39.0	32.4	38.5
3a	4	22.5	20.1	ND	ND	19.3	25.5
b	6	19.0	20.8	** -	-	ND	20.3
4	10	26.3	26.0	18.0	21.0	20.0	-
5	11	19.4	21.6	23.6	28.0	28.0	-
6	2	22.0	24.0	28.0	22.2	19.5	-
7	4	25.2	23.6	17.4	21.0	21.0	-
8	4	18.8	21.0	39.0	23.6	21.0	-
9	1	21.6	24.2	22.6	22.6	21.0	-

TABLE 3.10. Concentrations of LH-RF in plasma prior to and after coital activity in cannulated male rats.

* N.D. No LH-RF detectable in sample.

** No sample.

80 pg/ml, and the 90 % intercept was 18.0 pg/ml. The measurements in the remaining experiments were made in Assay No. 13, in which the 50 % intercept was 67 pg/ml, and the 90 % intercept was 12.0 pg/ml.

3.10. DISCUSSION

The first RIA for LH-RF was that of Kerdelhue and Jutisz (1972) reported in greater detail by Jutisz & Kerdelhue (1973). Specific antisera were generated in guinea pigs using synthetic, non-conjugated decapeptide, and used at a final dilution of 1:120. In this assay, capable of measuring amounts of LH-RF between 0.01 and 1.0 ng, the slope of the curve obtained with extract of ovine stalk-median eminence was parallel to that of the standard preparation and no cross reaction was observed with oxytocin, lysine-vasopressin, synthetic TSH-RF, ovine LH, FSH, and prolactin. They were unable to detect LH-RF in plasma from the normal ram and normal male rat. However, amounts of LH-RF up to 6 ng/ml of plasma were found in the normal ewe at the time of the preovulatory surge of LH and FSH. Plasma from hypophysectomised male and female rats contained about 4 ng/ml plasma. They discount any interference from plasma constituents in their assay, with the comment that when different doses of LH-RF were added to normal male rat plasma, an identical dose response curve to that of LH-RF dissolved in buffer was obtained. No dose range is given for this experiment however.

The RIA of Jeffcoate et al. (1973) first reported by Barker, Isles, Fraser and Gunn (1973) employed an antiserum raised in rabbits after synthetic LH-RF was conjugated to bovine serum albumin. In a report on its specificity (Jeffcoate, Holland, Fraser & Gunn, 1974) it is pointed out that their earlier antisera, which were of low titre and avidity, were raised against the desamido-decapeptide conjugated through the C-terminal glycine to bovine serum albumin. In later attempts to raise antisera, bovine serum albumin was conjugated by the carbodiimide reaction to the hydroxyl group of tyrosine. This antiserum however, which was of higher titre and avidity, bound the octapeptide (LH-RF 3-10) with an avidity equivalent to that with which it bound the decapeptide (LH-RF). This RIA was used to determine LH-RF concentrations in the sheep at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973) and in the rat on the afternoon of pro-oestrus (Fraser, Jeffcoate, Holland & Gunn, 1973). In the sheep, methanol extracted plasma LH-RF concentrations rose during oestrus from baseline levels of less than 10 pg/ml to peaks ranging between several hundred pg/ml to more than 10 ng/ml in two ewes, several such peaks being observed over the 10 hour time period during which plasma LH concentrations were raised in the same animals. In marked contrast, Nett, Akbar and Niswender (1974) found with their RIA method (Nett et al., 1973) that LH-RF levels ranged from 15 to 96 pg/ml during the preovulatory surge of LH in ewes, and that LH-RF levels were not significantly elevated when LH levels were high.

In the rat, on the afternoon of pro-oestrus, Fraser et al. (1973) found LH-RF concentrations of from less than 10 to 900 pg/ml in methanol extracted plasma. Highest values for serum LH-RF were found just before and during the ovulatory surge of LH observed in the same animals. Single observations were made on each animal, and blood was collected under ether anaesthesia. In a further report, Jeffcoate, Fraser, Holland & Gunn (1974) point out that their antisera (including an antiserum raised in rats against the octapeptide, LH-RF 3-10) are only specific for the C-terminal amino acid residues of LH-RF. They also indicate that serum does not itself interfere with the RIA since standard curves in serum previously "stripped" of LH-RF by charcoal are identical with those set up in buffer. In measurements made on human serum (Jeffcoate, Greenwood & Holland, 1974) using antiserum raised in rabbits, basal concentrations of LH-RF were less than the minimal detectable concentration in the assay, 10 pg/ml.

The fact that the antisera used by Jeffcoate and his colleagues are only specific for the C-terminal octapeptide sequence (LH-RF 3-10) calls into question the results outlined above. Furthermore, in their recent report (Jeffcoate, Holland, Fraser & Gunn, 1974) in which several synthetic fragments of LH-RF have been tested for their ability to displace ^{125}I-LH-RF from their antisera raised in rabbits, a figure appears demonstrating that fragments 7-10, 1-8, 1-9, 1.2-9.10, 3-9 and 1-6 of the LH-RF decapeptide exhibit displacement curves parallel to

that of decapeptide, albeit at concentrations 10 to 10,000 fold greater than those for the decapeptide and the octapeptide LH-RF 3-10.

The RIA of Arimura, Sato, Kumasaka, Worobec, Debeljuk, Dunn & Schally (1973) in its traditional competitive binding form appears to be specific for the decapeptide LH-RF. The fact that binding of labelled LH-RF was enhanced when small amounts of unlabelled hormone was added to incubation tubes (the so called "paradoxical"form of binding) however, was of some concern to these authors who were unable to offer any explanation for it. Nevertheless, they have used this "pardoxical" assay for determinations of LH-RF concentration in plasma from women in the reproductive age group at the time of the ovulatory surge of LH (Arimura, Kastin & Schally, 1974), finding LH-RF levels of up to 17 pg/ml at this time.

The assay of Nett et al. (1973) described in the present studies appears to be specific for the decapeptide, since none of the synthetic fragments described in Section 3.5 were capable of displacing ^{125}I-LH-RF from antiserum at concentrations ranging from 15 to 1000 pg/ml However, the free acid derivative and analogues of LH-RF modified at the glycine terminus did cross-react to a variable extent (Nett et al., 1973)

It is also clear that it is appropriate to measure LH-RF concentrations with this RIA in a number of biological materials, since a number of LH-RF containing materials exhibited inhibition curves

parallel to that of the standard curve. Regrettably, clear data on between-assay variability is lacking in the assays described. The quality control parameters listed, however, suggest that the assay is reasonably "in control", nearly all the values displayed in Figure 3.15 lying within the 95 % confidence limits.

The fact that intact plasma contains constituents which affect binding of hormone to antiserum means that care must be taken when interpreting results of determinations made with plasma volumes in assay incubation tubes greater than 100 µl, especially when the concentration of LH-RF in the sample is low.

The results of determinations of LH-RF concentration in post-menopausal women before and after treatment with oestrogen are difficult to interpret, particularly because the measured levels are close to the minimal detectable level of LH-RF in the assay, and also because estimations were carried out on 200 µl samples. It seems possible that the measured concentrations of LH-RF in these samples are underestimates, in view of the capacity of plasma to interfere with binding noted previously. It might be possible to discover measurable levels of LH-RF in post-menopausal plasma by extraction and deproteinisation of larger volumes of serum. Seyler & Reichlin (1973) have used an in-vivo bioassay method using OOP rats to measure LH-RF in large (40 ml) methanol extracted plasma samples from post-menopausal women. They claimed that LH-RF was detectable in the blood of some, but not

all post-menopausal women tested. Cyclic variations in the elevated LH levels of post-menopausal women (Yen, Tasi, Naftolin, Vandenberg & Ajabor, 1972; Root, Decherney, Russ, Duckett, Garcia & Wallach, 1972) have suggested to several authors (Yen et al., 1972; Malacara, Seyler & Reichlin, 1972) that LH-RF is secreted in periodic bursts in the absence of oestrogen secretion by the ovaries.

From the results of the present studies, it is impossible to comment on whether the oestrogen implants in those patients had any effect on the postulated hypersecretion of LH-RF in post-menopausal women.

It is apparent that coital activity in the male rat has no discernable effect on peripheral plasma LH-RF concentration over the time period of sampling in the experiments described here. Measurements were again made on single 200 µl samples, as successive blood sampling over a short period only allowed collection of 0.5 ml of blood per sample. Again, the fact that most of the LH-RF concentrations were close to the minimal detectable concentrations of LH-RF standard in the assay, and the effect of plasma on the assay, may mean that the estimations made are spurious. These investigations were undertaken in an attempt to demonstrate that LH-RF secretion is responsible for the significant rises in serum LH concentration found in male (and female) rats within 10 minutes of mating (Taleisnik, Caligaris & Astrada, 1966) although these findings have not been confirmed. Sexual stimulation

elicited rises in serum LH and serum testosterone in two artificial insemination donor bulls (Katongole, Naftolin & Short, 1971) although the assumption that these changes were directly related to sexual stimulation is open to question. In male rhesus monkeys, rises in plasma testosterone following sexual stimulation have been observed by Rose, Gordon & Bernstein (1972). In the human, urinary gonadotrophins increased, though hardly convincingly, following, and in anticipation of coitus in men (Farris, 1944; Masters, Magallon & Grody, 1952) and plasma testosterone levels appeared to be positively influenced by sexual activity in man (Fox, Ismail, Love, Kirkham & Loraine, 1972). Coitus in the rabbit appears to have no noticeable effect on plasma testosterone concentration (Dr. K. Brown-Grant and Miss F. Murray, personal communication).

If as suggested for the female by Crighton, Foster, Holland & Jeffcoate (1973) LH-RF secretion occurs in brief spurts in the male then it is conceivable that any rise in peripheral LH-RF concentration might only be discovered if continuous post-coital blood sampling were undertaken. On the other hand, any secretion of LH-RF in male rats during coital activity may have occurred either before or after the time periods studied in the present experiments. Confirmation of the work of Taleisnik, Caligaris & Astrada (1966) might encourage a resumption of the present studies.

CHAPTER 4

STUDIES ON HYPOPHYSIAL PORTAL BLOOD

4.1. SUMMARY

Luteinising hormone releasing factor can be detected and measured quantitatively in pituitary stalk plasma, although there are considerable discrepancies between results of in-vitro bioassay and radioimmunoassay. Stimulation of the preoptic area in male and female rats leads to increases in stalk plasma LH-RF content. Ovariectomised rats do not appear to have elevated secretion rates of LH-RF. There is some evidence that LH-RF secretion does not vary strikingly throughout the oestrous cycle "in phase" with LH secretion from the anterior pituitary gland.

4.2. INTRODUCTION

The establishment of in-vitro bioassays and a radioimmunoassay for LH-RF has made possible the quantitative investigation of its presence in pituitary stalk plasma. In the paragraphs that follow, experiments are described in which the concentration of LH-RF is determined in stalk plasma collected from animals subjected to a number of treatments, including electrical stimulation of the diencephalon.

4.3. HISTORY

The observation made in 1935 by Houssay, Biasotti & Sammartino, that in the toad, blood from the ventral hypothalamus

flowed towards the anterior pituitary did not receive wide attention, and the views of Popa and Fielding (1930, 1933) that the flow of blood in the "group of thin walled veins which collect blood from all parts of the hypophysis . . . . . and terminate in a network of fine vessels beneath the infundibular recess of the third ventricle" was from the pituitary towards the hypothalamus held sway until 1936. Pietsch (1930) also studied these vessels without attaching any particular significance to them. In 1936 Wislocki and King compared the pituitary portal system with the hepatic portal system and concluded that the vessels in the stalk represented the primary plexus from which blood flowed towards the pituitary along the portal veins to enter the pituitary sinusoids. Wislocki (1937) produced further evidence for his hypothesis from his investigations into the development of the blood supply of the hypophysis in the human.

In 1947, Green reported on his studies of several amphibian species. Studying the portal vasculature in living animals, he found the blood flow to be caudally directed. With Harris (Green & Harris, 1949) he observed for the first time the flow of portal blood towards the pituitary in an anaesthetised mammal, the rat. Similar observations were made by Barrnett & Greep (1951) and Daniel & Prichard (1956). Török (1954) in the dog and cat, Worthington(1955, 1960) in the mouse, and Daniel, Hitchcock, Pratt & Trieb (1961) in the Rhesus monkey and the baboon also observed the flow of blood in the portal vessels.

It was clear from direct observation that the flow of blood in the portal vessels was from hypothalamus to hypophysis. Other evidence, obtained by injection methods and histological examination supported this view. Green (1948) observed that when Indian ink was injected into the arterial system of dead rats the portal vessels filled from the hypothalamic end, and Morato (1939) showed that small fat emboli became lodged in the capillary plexus of the infundibulum and pars tuberalis at the cephalic end rather than at the caudal end of the system. In a survey of vertebrates, Green (1951) found portal vessel systems to be a constant feature from anurans to primates, and that cyclostomes, holostei, teleostei, elasmobranchii, necturi and urodela exhibited similar if less clear cut vascular arrangements. Wingstrand (1951) confirmed these findings for several species of birds.

As has been pointed out previously, it was concluded that these portal vessels carried humoral agents, now known and identified in some cases as releasing factors whose function is to transduce neural activity in the ventral diencephalon into biochemical responses by stimulating the synthesis and/or secretion of hormone(s) from the adenohypophysis.

The anatomy of this vital region bears closer examination. In the nomenclature of Rioch, Wislocki & O'Leary (1940) the neurohypophysis is divided into three parts, the neural lobe or infundibular process, the neural stalk or infundibular stem, and the median eminence or tuber cinereum. These parts show similarities which distinguish them

from the tissues of the closely adjacent hypothalamus in vascular supply (Wislocki, 1937; Green & Harris, 1947), cytology (Gersh, 1939; Weaver & Bucy, 1940), embryology (Tilney, 1936), affinity for various circulating hormones (Ford & Gross, 1958a, b; Ford, Kantounis & Lawrence, 1959; Taurog, Harris, Tong & Chaikoff, 1956) and high content of histamine (Harris, Jacobsohn & Kahlson, 1952) and catecholamines (Dahlstrom & Fuxe, 1966).

Green, in 1951, defined the median eminence as "that part of the neurohypophysis which receives its blood supply from the hypophysio-portal circulation, or which has a common vascularisation with the adenohypophysis." He suggested that the median eminence was phylogenetically the oldest part of the neurohypophysis and that the whole neurohypophysis of forms lower than the Amphibia corresponded to the median eminence of higher phyla. In Amphibia, Reptilia and Mammalia, the size of the neurohypophysis varies with the degree to which the animal depends on water in its environment. In these higher forms, Green divided the neurohypophysis into only two basic parts; the median eminence as defined above, and the neural lobe independently supplied with blood. The term neural stalk or hypophysial stalk is purely descriptive.

Now if, as Green suggests, the neural lobe is an evolutionary derivative from the median eminence, and since the neural lobe secretes peptide hormones such as oxytocin and vasopressin, then the supposition

that the median eminence might be similarly able to secrete peptides seems reasonable. It is important to continually recall that the median eminence is neurohypophysial in origin. One part of the neurohypophysis (the neural lobe) secretes peptide hormones into the general circulation to influence distant target organs such as the breast, uterus, and kidney. The other part (the median eminence) secretes peptide hormones into a local vascular circuit to influence the activity of the anterior pituitary gland which then itself influences distant target organs.

The median eminence, then, is the site where hypothalamic nerve terminals are likely to come into contact with the primary capillaries of the portal vasculature. Investigation of the median eminence by light microscopy is unrewarding because of the fine and unmyelinated character of nerve fibres in the ventral hypothalamus. Investigation with the electron microscope by many workers has thrown light on the neurovascular relationships. In the rat, Kobayashi Kobayashi, Yamamoto & Inatomi (1963), Bradbury & Harris (1964), Rohlich, Vigh, Teichmann & Aros (1965), Kobayashi, Oota, Uemura & Hirano (1966), Monroe (1967), Kobayashi & Matsui (1969), Fink & Smith (1971) and Monroe, Newman & Schapiro (1972) have all described the ultrastructure of the median eminence.

In the external layer of the median eminence axon terminals and ependymal processes abut onto the fenestrated capillaries, a

1000 Å gap intervening filled with collagen and bounded by two basal laminae. The axon terminals contain both small electron-lucent vesicles (synaptic-like, but there are no typical synaptic membrane thickenings) and larger dense-cored granular vesicles ranging from 500-1500 Å. There is a high concentration of mono-aminergic nerve terminals in this area (Carlsson, Falck & Hillarp, 1962).

The ependymal processes (the parent cell bodies line the floor of the III ventricle) contain fibrils, microtubules, glycogen, lipid and a variety of inclusions, including coated vesicles and granules.

Deep to this external layer lies a mass of axon, glial and ependymal elements (called the palisade layer by some authors), the axons containing considerable numbers of dense-cored vesicles and far fewer synaptic-like vesicles. The ependymal processes are extensively interdigitated with axons, synapse-like contacts between the two occurring frequently.

The evidence thus far presented, and additional evidence from experiments involving lesions of the hypothalamus, section of the pituitary stalk and transplantation of the pituitary gland to distant sites made it seem likely that the three link chain, hypothalamic nerve fibres, median eminence, hypophysial portal vessels, formed the essential pathway for control of anterior pituitary

function, and that specific releasing activity was to be sought in extracts of the median eminence and of portal vessel blood.

With the establishment of the hypophysial portal vasculature as the major supply route to the anterior pituitary, attempts to collect blood from the portal veins began with the work of Porter and his co-workers (Porter & Jones, 1956; Porter & Rumsfeld, 1956; Rumsfeld & Porter, 1959; Porter & Rumsfeld, 1959). They collected hypophysial portal blood from dogs by exposing the pituitary region by a temporal approach, removing the pituitary by suction and collecting blood from the cavity created by the displaced pituitary. Approximately 10 ml of plasma was collected over a 2 hour period. Such plasma was extracted by several methods and assayed for corticotrophin releasing activity by intravenous injection into rats and subsequent measurement of adrenal ascorbic acid depletion. They reported that plasma collected from the pituitary stalk caused a reduction of adrenal ascorbic acid in intact rats, no such reduction being found with carotid artery plasma samples collected from the same dogs. No adrenal ascorbic acid depletion was achieved when portal plasma was injected into hypophysectomised assay rats.

The blood Porter and his colleagues collected consisted of a mixture of blood from the hypophysial portal vessels, the veins and sinuses draining the sella turcica and the hypophysial arteries, as well as some cerebrospinal fluid. The exposure approach is a highly

stressing one and possibly helped to overcome the dilution factors, since the donor animals must have been in a state in which (if the hypothalamo-pituitary axis were intact) high rates of secretion of ACTH might be expected. Porter's method, involving as it did the sacrifice of relatively large and expensive animals was not improved upon until 1966 when Worthington developed a technique for the collection of hypophysial portal blood from the cut pituitary stalks of rats. G.W. Harris (1971) in his Dale Lecture remarks that he and Donovan collected portal blood from cats in 1955-56 and assayed it, unsuccessfully, for catecholamine content. The method of Worthington (described in detail in Section 4.4) involves exposure of the pituitary stalk from the inferior aspect by removal of the basis-phenoid bone. The stalk is cut and blood continuously sucked from the cut end through capillary tubing. The "blood" collected is a mixture of portal blood, cerebrospinal fluid, blood flowing retrogradely from the pituitary sinusoids, and often (because they lie close alongside or in the pituitary stalk in the rat) blood from small branches of the anterior hypophysial arteries as well. While the portal vessels are probably the most important source of blood passing the anterior pituitary (Lascano-Gonzales, 1935; Daniel, Prichard & Schurr, 1958) there are undoubtedly direct contributions from the anterior hypophysial arteries.

Worthington (1966) reported that the volume of blood collected over a one hour period was 1.4 to 2.2 ml. Data from two

different laboratories (Goldman, 1963; Porter, Hines, Smith, Repass & Smith, 1967) suggest that the rate of flow of blood through the pituitary stalk vessels is approximately 0.4 ml/hour. It may be that resistance to blood flow in the portal vessels falls if they are transected at the stalk/pituitary junction. This would increase the volume of "portal blood" collected in a given time. Even so, it must be admitted that collections made by Worthingston's method do not consist of pure portal blood. In particular, it must be remembered that retrograde flow through pituitary sinusoids will no doubt contribute adenohypophysial hormones to the collection.

In 1967, Porter and Smith described a different procedure for collecting portal blood from the anaesthetised rat. They exposed the pituitary by a parapharyngeal approach as for hypophysectomy, removing the bone and dura overlying the ventral surface of the gland. The veins draining the gland are ruptured, the stalk transected and the entire pituitary removed. This leaves a stump of pituitary stalk protruding into the pituitary fossa. After heparinising the animal, a plastic tube is placed over the stalk and applied to the diaphragma sellae forming a liquid tight seal. Negative pressure is then applied to the cannula and blood collected for up to 6 hours, the collection rate being 6 µl/min.

It is claimed that the blood so collected is pure portal blood. Porter, Goldman & Wilber (1970), in putting this claim forward,

make no reference to branches of the anterior hypophysial arteries running in the stalk. Porter et al. (1970) criticise the method of Worthington not only because of the "impurity" of the portal blood collected by his technique but also because the exposed pituitary stalk and median eminence are likely to dry up. This is not so. Blood pooling around the median eminence and stalk keep the whole area moist. In addition, exposure of the entire area allows periodic inspection of the median eminence and stalk for evidence of portal vessel thrombosis and oedema of the median eminence, which occur on infrequent occasions. Porter and Smith's method does not allow of any such direct observation, though they suggest that at the end of portal blood collection the stalk "looks quite normal" (Porter et al., 1970). The application of plastic tubing to such delicate tissue as the pituitary stalk would seem to increase the likelihood of trauma however.

The major advantage claimed (Porter et al., 1970) for the collection method of Porter and Smith is that as the animal is hypophysectomised prior to collection of blood, the admixture of pituitary sinusoid blood containing adenohypophysial hormones is avoided. However, Midgley (1966) has demonstrated by immunohistochemical techniques the presence of LH in the pars tuberalis of the human, and Porter et al. (1970) find higher concentrations of LH in their portal blood collections than in peripheral blood from the same animal. An additional advantage claimed for the method of

Porter and Smith is that no cerebrospinal fluid contaminates the collection.

Demonstration of the presence of LH-RF activity in portal blood was first made by workers in Oxford (Fink, Nallar & Worthington, 1966, 1967; Fink, 1967a, 1967b) using the OAAD method for assaying LH-RF. They found that pituitary stalk plasma obtained from female rats during pro-oestrus caused a significantly greater OAAD than did peripheral plasma from the same animals. In a further experiment, pituitary stalk plasma from hypophysectomised rats caused greater OAAD than did peripheral plasma. The central problem in the interpretation of these results was whether the OAAD induced by stalk plasma was due to LH-RF, or rather due to LH present in the stalk plasma. Retrograde flow from the pituitary may contain considerable quantities of adenohypophysial hormones, as was demonstrated by Averill, Salaman & Worthington (1966) who assayed stalk plasma for thyroid stimulating hormone (TSH). The concentration of TSH was considerably higher than that in peripheral plasma from the same animals. Fink et al. (1967) argued that their experiments on hypophysectomised animals made it seem unlikely that stalk plasma was contaminated by LH, and that therefore their results provided strong evidence for the existence of LH-RF in portal blood.

In further experiments (Fink & Harris, 1970), attempts were made to exclude the possibility of contamination with LH, by

subjecting portal plasma to various extraction procedures. Both acid ethanol and acetic acid extraction were found to be capable of inactivating LH, the OAAD assay being used to test the extracts. Extracts of saline homogenates of rat adenohypophyses, and of stalk plasma from hypophysectomised or ovariectomised rats was subjected to fractionation on Sephadex G-25, which has an exclusion limit of 5000. This produced two main fractions, Fraction I which contained substances excluded by the gel, and Fraction II which contained substances retarded by the gel. Fraction I from all these sources, as well as from extracted systemic plasma caused OAAD, an observation which Fink and Harris (1970) attributed to non-specific OAAD activity of plasma proteins similar to that described by Pelletier (1965), and not to LH or LH-RF. Fraction II of stalk plasma from ovariectomised or hypophysectomised rats elicited a higher degree of OAAD than did the same fraction of systemic plasma. Similarly, using OOP rats as the test animals for the OAAD assay, it was found that Fraction II of stalk plasma from ovariectomised rats caused significant OAAD while Fraction II of systemic plasma did not. When infused directly into the anterior pituitary gland of rabbits, Fraction II of stalk plasma from ovariectomised rats caused ovulation. Again, Fraction II of systemic plasma did not.

Subsequently (Burger, Fink & Lee, 1972) gel-fractionation was discarded in favour of the ultrafiltration described in the

present studies. Only a trace of LH (measured by RIA) was present in ultrafiltered extracts of stalk and systemic plasma from ovariectomised rats. Both stalk and systemic plasma exhibited LH-RF activity; the assay system employed OOP rats.

Other work on LH-RF in portal blood has been carried out by Porter and his co-workers (Kamberi, Mical & Porter, 1969, 1971; Ben-Jonathan, Mical & Porter, 1973). They have employed a superfusion technique in which one hemipituitary is superfused with portal blood, the other with arterial blood from the same animal. Portal blood from ovariectomised rats caused release of LH in this assay system at a rate twice that caused by arterial blood from the same animals. By contrast, there was no difference in rates of LH release when portal and arterial blood from dioestrous animals was tested. In earlier studies (Kamberi et al., 1970), it had been shown that portal blood from normal male animals was capable of releasing FSH from hemipituitaries incubated in-vitro, while arterial blood had no such effects.

The investigations described above suggested that portal vessel blood did contain substances capable of causing the release of gonadotrophins from the anterior pituitary gland.

The further investigation of the existence of LH-RF in pituitary stalk plasma to be described in the present studies was undertaken in the light of an important advance, namely the

characterisation and synthesis of LH-RF. This meant that quantitative bioassay and radioimmunoassay of LH-RF became available, affording opportunities for closer and more detailed study of the hypothalamo-pituitary axis with respect to the control of gonatotrophin secretion.

4.4. Experimental Methods

Animals. Male and female rats of the Wistar strain (Charles River (UK) Ltd; Margate) weighing 220-400 g were kept under controlled lighting conditions (14 h. light, 10 h. dark) and temperature (22° C) and fed diet 41B pellets (E. Dixon & Sons Ltd.; Ware), and tap water ad libitum. Animals (220-280 g) bearing chronic electrode implants were provided with drinking water containing chlortetracycline (Aureomycin, Cyanamid; London) 40 mg/l.

The oestrous cycles of rats were followed by daily microscopic examination of vaginal smears. These were obtained by lavage with 0.9% saline and inspected within 10 min. The days of the cycle (4 day) were identified as follows:

Dioestrus - leucocytes with occasional epithelial cells,

Pro-oestrus - nucleated epithelial cells,

Oestrus - cornified epithelial cells,

Metoestrus - leucocytes and both cornified and nucleated epithelial cells.

The assistance of Dr. G. Fink with smearing and examination

of smears is gratefully acknowledged.

In female animals which were to be electrically stimulated, at least two consecutive 4-day cycles were observed prior to electrode insertion on the afternoon of dioestrus. Following recovery, a further two consecutive 4-day cycles were observed prior to stimulation and collection of portal blood.

Electrodes. Twin unipolar platinum electrodes as described previously were implanted either acutely or chronically into the preoptic areas of male rats, and chronically into female rats. The procedure was as described previously, except that steel screws (10 BA, 3 mm, Walker-Spencer; Birmingham) were inserted into holes drilled in the parietal bones on each side at their postero-lateral aspects, and into the interfrontal suture anteriorly. These screws provided anchorage for the acrylic cement (Simplex, Dental Fillings Ltd; London) which was used to anchor the electrode assembly in place. The skin flaps were approximated over the assembly with everting sutures. Infection, either intra- or extra-cranial was experienced in 5% of animals thus treated. A diagram of stimulation areas appears in Appendix 2.4.

Collection of portal blood. Pituitary stalk blood was collected according to the method of Worthington (1966). Each animal was anaesthetised with Urethane 1g/kg in a 10% solution injected intra-peritoneally. Urethane provides a satisfactory level of anaesthesia forlong periods (in excess of 4 h.). Worthington (1966) has observed

that sodium pentobarbitone anaesthesia reduces portal blood flow in mice, and Fink (1967a) has confirmed this effect in rats. The animal was immobilised supine on an operating board and a midline incision was made in the skin of the lower jaw extending from the lips to the sternal notch. Guy-sutures were inserted into the flaps of the divided lip and into the tip of the tongue. The pretracheal muscles were divided transversely and the trachea intubated via a transverse incision with a polythene tube (1.5 cm long, Portex PP 205), which was tied in place. A tight ligature was placed around each mandibular ramus anterior to the molar teeth, and the lower jaw divided in the midline. The muscles of the floor of the mouth were divided on each side sufficiently far posteriorly to expose the soft palate and epiglottis. Bleeding was controlled with traction on the tongue. The tongue and the floor of the mouth were then carried caudally and fixed in place under slight tension. Oxidised cellulose gauze (Ethicon; Edinburgh) served to control haemorrhage from cut surfaces.

The soft palate was incised with an eye cautery from the posterior aspect of the hard palate to the epiglottis, and laterally to the inferomedial aspects of the pterygoid processes of the basisphenoid. The mucosa of the roof of the nasopharynx was detached from the inferior surface of the basisphenoid with pledgets of cotton-wool.

At this point the right external jugular vein was exposed by blunt dissection. From this point on, the procedure was carried out with the aid of a binocular operating microscope (Carl Zeiss; Germany). With a dental drill, the outer table of the basisphenoid was removed from the occipito-sphenoid suture to the basisphenoido-presphenoid suture anteriorly. From time to time, the hole was packed with bone wax (Ethicon; Edinburgh), particularly in the vicinity of the transverse venous sinus. The inner table was then eburnated until it yielded to gentle pressure with a dental pick. The thin plate of bone was carefully freed from the underlying dura mater. Bleeding from dural vessels was controlled with cotton-wool pledgets. When haemostasis had been secured the dura mater was incised on either side with a fragment of safety razor blade (Blue, Gillette; London) held in a pin chuck. The flap of dura mater was retracted caudally, followed by the arachnoid mater. The portal vessels could now be observed. The animal was then given an intravenous injection of 500 IU Heparin (Pularin, Evans Medical; Liverpool) in a volume of 0.1 ml via the external jugular vein. Insertion of the needle through the pectoral muscles prevented haemorrhage.

The pituitary stalk was then cut transversely at its junction with the anterior pituitary gland with iridectomy scissors (Weiss; London).

Blood was collected from the anterior end of the trough in

the sphenoid bone either by continuous suction through a vinyl tube (O.D. 0.9 mm, Becton-Dickinson; New Jersey) directly into ice-cooled centrifuge tubes or by periodic transfer of pooled blood into ice-cooled centrifuge tubes with a pasteur pipette. With both collection methods periodic observation of the stalk and vessels could be made. Collection via vinyl-tubing caused (on inspection) a much greater degree of haemolysis than did periodic removal.

At the end of the collection the sample was centrifuged at room temperature for 10 minutes (Minor, MSE; London). The plasma was stored at -15° C until used.

The heads of animals bearing electrodes were fixed in buffered formalin, and thionin stained 40 µm frozen sections prepared.

Extraction of LH-RF from Portal Plasma. Ultrafiltrates of plasma were prepared according to the method of Burger, Fink & Lee (1972). Plasma was extracted on a flask-shaker (Griffin; Wembley) with five volumes of acid ethanol, pH 1.75 (12N HCl, absolute ethanol, distilled water : v/v, 0.5, 75, 25) for 30 min. The material was centrifuged at 12,500 G ("Superspeed", MSE; London) for 30 min. at 4°C. The precipitate was resuspended in 5 volumes of acid ethanol, shaken for 30 minutes and centrifuged as before. The supernatants were combined and evaporated to dryness in a rotary film evaporator (Buchi; Switzerland)

at a temperature of 30-35°C. The resulting film was resuspended in a volume of distilled water equivalent to twice the volume of the original plasma and subjected to ultrafiltration at 4°C under nitrogen at 34.5×10^4 $N.m^{-2}$ using a Diaflo apparatus (Amicon Corporation; Mass.) and Diaflo UM-10 filters (retentivity, MW >10,000). The ultrafiltrate was lyophilised (Quickfit; Staffs.) and stored at -15°C.

Immediately prior to assay of the portal plasma extract, the lyophilate was resuspended in 2-4 ml. distilled water and brought to pH 7.0 assessed with full-range indicator paper (Whatman-BDH; Poole) by dropwise addition of ION. NaOH solution.

Characterisation of Cardio-respiratory Responses to Electrical Stimulation of the Diencephalon. Rats bearing chronic electrode implants were anaesthetised with urethane 1g/kg and immobilised supine on an operation board. A cannula filled with saline containing 100 i.u. heparin/ml. was inserted into the right femoral artery (Portex, PP00). The other end was connected to a pressure transducer (P 23 AC, Statham, Sensitivity range 0-75 cm. Hg) coupled to a low level d.c. preamplifier and pen recorder (Model 4, Gross). A further cannula (Portex, PP205) with multiple perforations in its terminal 1" was inserted into the oesophagus, the other end (air-filled) being connected to a pressure transducer (P23BC, Statham, Sensitivity range 0-5 cm. Hg) coupled to the pen recorder.

Recordings were made during and after periods of stimulation, and after i.p. administration of atropine (BDH; Poole) 0.5 g/kg, i.v. vasopressin (Parke-Davis; USA) 40 mu/kg, i.v. propanolol (ICI; Macclesfield) 4 mg/kg, i.v. phentolamine (Ciba; London) 4 mg/kg or i.v. phenoxybenzamine (Smith, Kline and French; Herts) 10 mg/kg in 0.9% saline in a volume of 0.1 - 0.2 ml.

At the end of experiments the heads were fixed in 10% buffered formalin and thionin stained 40 µm frozen sections prepared.

4.5. RESULTS

Measurement of LH-RF in pituitary stalk plasma by in-vitro bioassay.

The investigations reported in Chapter 2 prompted the examination of stalk plasma collected during electrical stimulation of the medial preoptic area of male rats. These studies were undertaken before the completion of experiments on parameters of stimulation, so that collections of stalk blood were not necessarily made during the application of the most effective stimulus parameters.

Male Assay System. Stalk blood was collected from male rats bearing twin unipolar platinum electrodes implanted acutely into the medial preoptic area. Blood was collected for one hour periods, the stimulus parameter being biphasic pulses, 50 Hz, pulse amplitude 0.5 mA, pulse

duration 2 msec., 30 sec on/off. The results of two assays in which ultrafiltrates of stalk plasma were tested for LH and FSH releasing activity appear in Table 4.1. In assay 2, no control flasks were included and therefore observations derived from it must be tentative. The slopes of dose-response lines calculated are significantly different from one another and calculation of potency has been made with one dosage level of unknown. The stalk ultrafiltrate is more potent with respect to LH release than to FSH release and appears to contain enormous quantities of material with LH-RF like activity.

In assay 3, slopes of dose-response lines were not significantly different from one another and potency calculations could be made on a 2 + 2 basis. The stalk ultrafiltrate is again more potent with respect to LH release than to FSH release. The amounts of LH-RF in stalk ultrafiltrate collected over a half-hour period are 30 ng with respect to LH release and 16 ng with respect to FSH release.

The concentration of K^+ in media from flask to which ultrafiltrate had been added ranged from 12 - 14 mM, a level which had no effect on either LH or FSH release. Neither LH, nor FSH could be detected in ultrafiltrates by RIA.

Female Assay System. Stalk blood was collected from male rats bearing unipolar platinum electrodes implanted acutely into the

		LH-RF ng/ml medium				STALK ULTRAFILTRATE min/ml medium				
Assay No.	Control	2.0	8.0	λ	F	4.4	17.5	λ	F	
2										
LH ng/ml medium	-	615 ± 96	1203 ± 82	0.18	22 p <0.01	814 ± 57	2136 ± 315	0.20	17 p <0.01	Combined F = 4.53 slopes significantly dif Potency (2 + 1) M = 70.8 ng ie.121ng/30m
		p <0.01 y = 979x + 322				p <0.001 y = 2203x + 153				
FSH ng/ml medium	-	11620 ± 870	17330 ± 1100	0.21	17 p <0.01	15280 ± 920	23750 ± 2600	0.38	9 p <0.05	Combined - Potency (2 + 1) M = 37 ng ie. 63ng/30min.
		p <0.01 y = 9500x + 8780				p <0.05				
	Control	2.0	8.0		F	5	20		F	
3	491 ± 20	*926 ± 89	1743 ± 35	0.23	13 p <0.05	*1145 ± 40	1713 ± 145	0.21	16 p <0.01	Combined F = 0.81 Potency (2 + 2) M = 20.0ng ie. 30ng/30min. Limits (12.0 - 39.0)
		p <0.01 y = 1326x + 502				p < 0.05 y = 974x + 533				
FSH ng/ml	7360 ± 630	*11500 ± 1140	17800 ± 1890	0.28	9 p <0.05	* 13100 ± 480	18450 ± 2020	0.30	7 p <0.05	Combined F = 0.11 Potency (2 + 2) M = 10.7 ng Limits (4.9-28.2) i.e. 16 ng/30 min.
		p <0.05 y = 11080x + 7790				p <0.01 y = 9410x + 9950				

*p <0.05 when compared with control.

TABLE 4.1 Male assay system. LH and FSH responses (mean + S.E.M.) to synthetic LH-RF and to ultrafiltrates of pituitary stalk blood collected during medial preoptic area stimulation.

medial preoptic area, and tested for LH-RF activity in assays 8, 11, and 14. Blood was collected for one hour periods, the stimulus parameters being biphasic pulses, 50 Hz, pulse amplitude 0.5 mA, pulse duration 2 msec., 30 sec. on/off.

For assay 21, stalk blood was collected from male rats bearing electrodes implanted chronically into the medial preoptic area. Blood was collected for two consecutive 30 min. periods, stimuli (biphasic pulses, 60 Hz, pulse amplitude 0.5 mA, pulse duration 1 msec., 30 sec. on/off) being passed in the second 30 min. period only.

In all assays ultrafiltrates of stalk plasma caused release of both LH and FSH into incubation medium; the results appear in Table 4.2.

In assays 8, 11, and 14, calculations of potency of ultrafiltrate with respect to LH or FSH release could not be made on a 2 + 2 basis, either because the responses to the lower dose of ultrafiltrate were not significantly different from control values, or because the slopes of dose-response curves for standard and ultrafiltrate were divergent. Calculation with one dosage of stalk plasma ultrafiltrate gave amounts of 7.0, 3.8 and 1.1 ng LH-RF/30 min. for LH release, and amounts of 9.8, 5.0, and 0.3 ng LH-RF/30 min. for FSH release.

In assay 21, calculations of potency of ultrafiltrate with

TABLE 4.2

	ASSAY NO.	LH-RF ng/ml medium				STALK ULTRAFILTRATE min/ml medium				
	8	Control	7.5	30.0	F	4.5	18.0		F	
LH ng/ml medium		1210 ± 40	*2505 ± 135	4185 ± 310	0.20 p <0.01	20 p <0.01	1440 ± 125	*1750 ± 110	-	-
			p <0.01				p = ns			
			y = 2815x + 2780							
FSH ng/ml medium		4880 ± 240	*8430 ± 880	16870 ± 1900	0.23 p <0.05	14 p <0.05	4600 ± 400	*6750 ± 580	-	-
			p <0.01				p <0.05			
			y = 14240x - 4150							
	11	Control	3.75	7.5	F	5.0	20.0		F	
LH ng/ml medium		653 ± 80	*3750 ± 505	6000 ± 700	0.12 p <0.05	10 p <0.05	*1170 ± 150	2095 ± 250	0.11 p <0.05	14
			p <0.05				p <0.05			
			y = 8595x - 1395				y = 3470x - 900			
FSH ng/ml medium		4320 ± 480	*9530 ± 520	15030 ± 1010	0.04 p <0.001	117	4740 ± 570	*7680 ± 740	-	-
			p <0.01				p <0.02			
			y = 21250x - 3230							

	Combined - Potency (2 + 1) M = 4.2 ng ie. 7.0ng/30min
	Combined - Potency (2 + 1) M = 5.9 ng ie. 9.8ng/30min.
	Combined F = 3.05 slopes significantly differ Potency (2 + 1) M = 2.5 ng ie. 3.8ng/30min.
	Combined - Potency (2 + 1) M = 3.3 ng ie. 5.0ng/30min.

.....Table 4.2 cont.

ASSAY No. 14	Control	LH-RF ng/ml modium 1.9	7.5	F	STALK ULTRAFILTRATE min/ml modium 5.0	20.0	F	
ng/ml edium	1150 ± 40	*4650 ± 340	8175 ± 820	0.21 16 p <0.01	1330 ± 110	*1910 ± 55	- -	Combined - Potency (2 + 1)
		p <0.01			p <0.05			
		y = 5810x + 3040						M = 0.7 ng ie. 1.1ng/30min.
I ng/ml edium	3930 ± 360	*10580 ± 770	13670 ± 700	0.35 6 p <0.1	4350 ± 330	*6280 ± 350	- -	Combined - Potency (2 + 1)
		p <0.05			p <0.01			
		y = 4410x + 9370						M = 0.2 ng ie. 0.3ng/30min.
21	Control	7.5	30.0	F	7.5	30.0	F	
					PRE-STIMULATION			
LH ng/ml medium	1220 ± 130	*2810 ± 260	7420 ± 370	0.09 98 p<0.001	1680 ± 360	*2920 ± 440	- -	Combined - Potency (2 + 1)
		p <0.001			p = ns			M = 7.9 ng ie. 7.9ng/30min.
		y = 7660x - 3910						
					STIMULATION			
					*1980 ± 100	4550 ± 540	0.18 21 p<0.01	Combined F = 8.0 slopes significantly differ
					p <0.01			Potency (2 + 1)
					y = 4260x - 1760			M = 12.9ng ie.12.9ng/30min.

....Table 4.2 cont.

ASSAY No.		LH-RF ng/ml medium				STALK ULTRAFILTRATE min/ml medium				
21	Control	7.5	30.0		F	7.5	30.0		F	
SH ng/ml medium	5910 ± 1140	*11890 ± 890	25250 ± 2040	p <0.01 y = 22250x - 7680	0.14 p<0.01	PRE-STIMULATION 8530 ± 1020	*10290 ± 460 p = ns	-	-	Combined - Potency (2 + 1) M = 6.5 ng ie. 6.5ng/30mi
						STIMULATION *10180 ± 360	15500 ± 1780 p <0.05 y = 8840x + 2410	0.29 p <0.05	8	Combined F = 7.8 slopes significantly diff Potency (2 + 1) M = 10.8 ng ie. 10.8 ng/ 30 min.

* p <0.05 when compared with control.

TABLE 4.2 Female assay system. LH and FSH responses (mean + S.E.M.) to synthetic LH-RF and to ultrafiltrates of pituitary stalk blood collected prior to (assay 21) and during medial preoptic area stimulation.

respect to LH or FSH release could not be made on a 2 + 2 basis, either because the responses to the lower dose of ultrafiltrate were not significantly different from control values (as in pre-stimulation plasma collection ultrafiltrates) or because the slopes of the dose-response curves for standard and ultrafiltrate were divergent (as in stimulation plasma collection ultrafiltrates). Calculation with one dosage of stalk plasma ultrafiltrate gave amounts of 7.9 and 12.9 ng LH-RF/30 min. for LH release in the pre-stimulation and stimulation periods respectively, and amounts of 6.5 and 10.8 ng LH-RF/30 min. for FSH release in the pre-stimulation and stimulation periods respectively.

The concentration of K^+ in media from flasks to which ultrafiltrate has been added ranged from 12-14 mM, a level which had no effect on either LH or FSH release. Neither LH, nor FSH, could be detected in ultrafiltrates by RIA.

Measurement of LH-RF in pituitary stalk plasma by radioimmunoassay.

A Male animals electrically stimulated in the medial preoptic area.

In view of the results of investigations reported in Chapter 2, and also of the results of measurement of LH-RF in stalk plasma ultrafiltrates just described, it was decided to examine pituitary stalk plasma for the presence of LH-RF prior to and during electrical stimulation of the medial preoptic area.

Present Experiments. Stalk blood was collected for two consecutive 30 min. periods from 21 male rats bearing twin unipolar electrodes implanted into the medial preoptic area. In ten experiments (control), no current was passed in either period; in eleven experiments current was passed in the second 30 min. period (parameters, biphasic pulses, 60 Hz, pulse amplitude 0.5 mA, pulse duration 2 msec., 30 sec. on/off). Concentrations of LH-RF in samples were determined in duplicate aliquots of 100 µl; the control samples were assayed in Assays 14 (50% intercept 105 pg/ml) and 18 (50% intercept 90 pg/ml), the remainder in Assay 12 (50% intercept 80 pg/ml).

The results of these experiments appear in Table 4.3 and Figure 4.1 and are summarised in Table 4.4. In eleven experiments in which current was passed, the volume of plasma (mean ± S.E.M.) collected in the first 30 min. was 0.5 ± 0.1 ml, and the concentration and total content (i.e. the product of concentration and plasma volume) were 40.6 ± 2.3 pg/ml and 18.2 ± 2.9 pg respectively. During the 30 min. period of stimulation the volume of plasma collected was 1.2 ± 0.2 ml, while the concentration and content of LH-RF were 74.3 ± 11.0 pg/ml and 83.9 ± 17.6 pg respectively. As determined by paired t test, the increments in plasma volume and in concentration and content of LH-RF which occurred during electrical stimulation were significant ($p < 0.001$, $p < 0.02$, $p < 0.01$ respectively).

In ten control experiments, the volumes of plasma collected

NO.	CONTROL (n = 10)						NO.	EXPERIMENTAL (n = 11)					
	Plasma Volume (ml)		LH-RF Concentration pg/ml		LH-RF Content pg/30min.			Plasma Volume (ml)		LH-RF Concentration pg/ml		LH-RF Content pg/30min.	
	A	B	A	B	A	B		C	D	C	D	C	D
1	0.50	0.70	39.5	26.0	19.8	18.2	1	0.75	1.30	47.0	69.0	35.3	89.7
2	0.45	0.60	34.5	26.0	15.5	15.6	2	0.30	0.70	44.5	75.0	13.4	52.5
3	0.60	0.80	26.5	28.0	15.9	22.4	3	0.40	1.50	47.0	71.0	18.8	106.5
4	0.80	0.40	42.0	31.5	33.6	12.6	4	0.70	1.70	36.5	137.0	25.6	232.9
5	0.70	0.60	45.0	32.5	31.5	19.5	5	0.35	0.45	37.0	45.0	13.0	20.3
6	0.75	0.85	74.0	63.0	55.5	53.6	6	0.50	1.80	48.0	44.5	24.0	80.1
7	0.40	0.20	70.0	63.0	28.0	12.6	7	0.80	1.60	41.0	62.5	32.8	100.0
8	1.40	1.00	30.0	24.0	42.0	24.0	8	0.30	0.40	27.2	56.0	8.2	22.4
9	0.40	0.40	49.0	28.0	19.6	11.2	9	0.40	0.60	31.0	152.0	12.4	91.2
10	0.25	0.40	41.0	51.0	10.2	20.4	10	0.25	0.70	51.5	56.0	12.9	39.2
							11	0.30	1.80	36.0	49.0	10.8	88.2
MEAN ± S.E.M.	0.63	0.60	45.2	37.3	27.2	21.0		0.46	1.14	40.6	74.3	18.2	83.9
	0.10	0.08	5.0	4.9	4.4	3.9		0.06	0.17	2.3	11.0	2.9	17.6

TABLE 4.3 Volumes, LH-RF concentrations and LH-RF contents of portal plasma collected from male animals bearing chronically implanted electrodes in the medial preoptic area. Two consecutive 30 min. collection periods; in the experimental group electrical stimulation occurred in the second 30 min. period only.

A = 0 - 30 min. (Control)

B = 30 - 60 min. (")

C = 0 - 30 min. (Experimental)

D = 30 - 60 min. (" , Stimulation period).

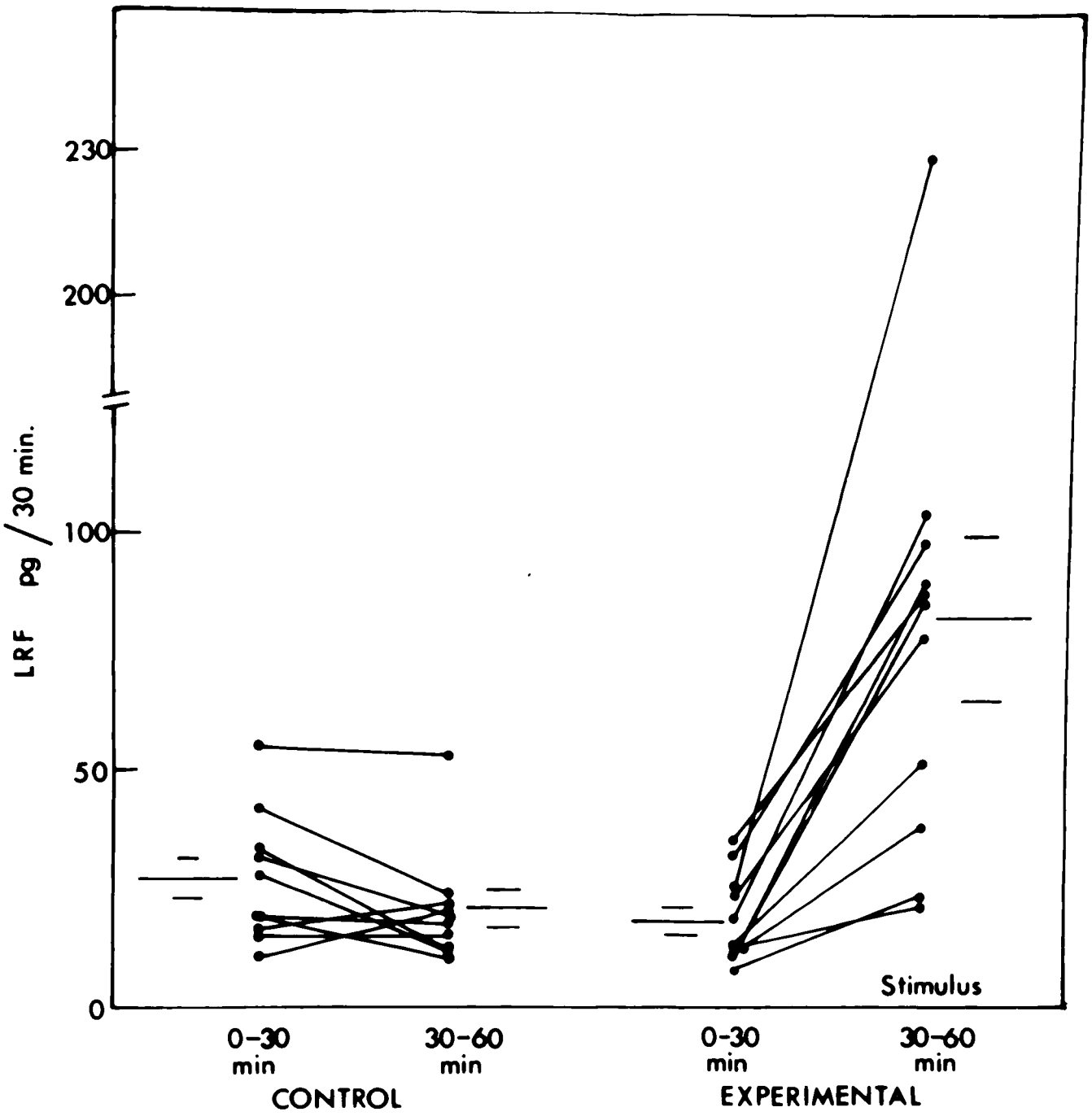


Figure 4.1 LH-RF contents of pituitary stalk plasma collected over two consecutive 30 min. periods in 10 control and 11 "experimental" male animals (see text).

	CONTROL n = 10		EXPERIMENTAL n = 11	
	1	2	3	4
Plasma Volume (ml)	0 - 30 min. 0.63 ± 0.10	30 - 60 min 0.60 ± 0.08	0 - 30 min. 0.46 ± 0.06	30 - 60 min. 1.14 ± 0.17 ** p < 0.001
LH-RF Concentration (pg/ml)	45.2 ± 5.0 *p < 0.05	37.3 ± 4.9 *p < 0.01	40.6 ± 2.3 *p < 0.01 ** p < 0.02	74.3 ± 11.0
LH-RF Content (pg/30 min.)	27.2 ± 4.4 *p < 0.01	21.0 ± 3.9 *p < 0.01	18.2 ± 2.9 *p < 0.01 ** p < 0.01	83.9 ± 17.6

TABLE 4.4 Summary. Volumes, LH-RF concentrations, and LH-RF contents of portal plasma collected from male animals bearing chronically implanted electrodes in the medial preoptic area prior to and during electrical stimulation.

* Significance when compared with column 4, unpaired t test.

** Significance between columns 3 & 4, paired t test.

(0.6 ± 0.1 and 0.6 ± 0.1 ml in the first and second 30 min. periods respectively) the concentrations of LH-RF (45.2 ± 5.0 and 37.3 ± 4.9 pg/ml in the first and second 30 min. periods respectively) and the contents of LH-RF (27.2 ± 4.4 and 21.0 ± 3.9 pg in the first and second 30 min. periods respectively) were not significantly different from those obtained in the period before the application of current in the experimental group.

B Female animals electrically stimulated in the medial preoptic area at various times during the oestrous cycle.

In female rats, there are two periods in the oestrous cycle at which there are increases in plasma LH concentration, one, the most marked, at pro-oestrus, and a second much smaller increase at metoestrus (Naftolin, Brown-Grant & Corker, 1972). In recent studies, the responsiveness of the hypothalamo-hypophysial axis to injections of synthetic LH-RF (Aiyer, Fink & Greig, 1974) and to electrical stimulation of the medial preoptic area (Fink & Aiyer, 1974) has been tested at various times throughout the oestrous cycle. Both for LH and FSH, the profiles of responsiveness throughout the oestrous cycle of the hypothalamo-hypophysial axis to electrical stimulation resemble closely the profiles of responsiveness of the anterior pituitary gland to LH-RF administered intravenously (Figure 4.2). There was a relatively gradual increase in pituitary sensitivity, in terms of the mean maximal increments of peripheral LH between the early afternoon

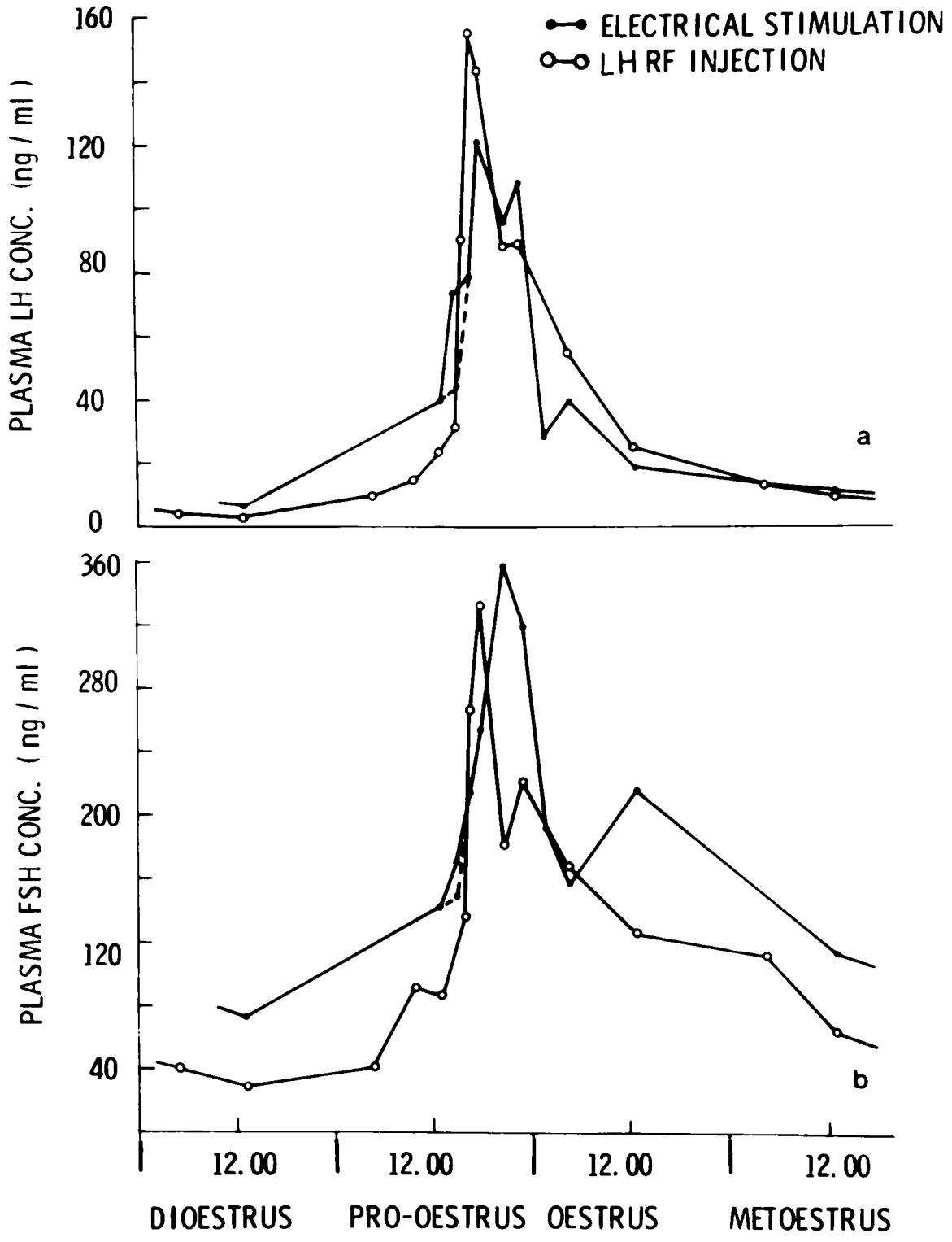


Figure 4.2 Comparison of the mean maximal increments in LH (a) and FSH (b) after electrical stimulation of the medial preoptic area or i.v. injection of LH-RF after anaesthesia at various times of the oestrous cycle. From Fink G. & Aiyer M.S. (1974) J. Endocr. In Press. By kind permission of the authors, and The Journal of Endocrinology.

of dioestrus and proestrus, followed by an abrupt and marked rise which reached a peak at 18.00 and 21.00 h. of pro-oestrus for LH and FSH, respectively. Sensitivity of the hypothalamo-hypophysial axis declined through oestrus and metoestrus, reaching a nadir at 13.30 h. of dioestrus.

The present experiments involving electrical stimulation of the medial preoptic area of female rats during the oestrous cycle were undertaken in order to ascertain first whether the increases in plasma LH concentration throughout the cycle were associated with increases in LH-RF secretion into portal blood, and secondly whether the sensitivity of the preoptic area - median eminence - portal vessel axis to electrical stimulation varied during the oestrous cycle.

Present Experiments. Stalk blood was collected for two consecutive 30 min. periods from 44 female rats bearing chronically implanted twin unipolar electrodes in the medial preoptic area. Stimulation was applied only in the second 30 min. period (parameters; biphasic pulses, 60 Hz, pulse amplitude 0.5 mA, pulse duration 1 msec., 30 sec. on/off). Stimulation was carried out at (No. of animals): 18.00 h. dioestrus (5), 09.00 h. (7), 13.00 h. (8) and 18.00 h. pro-oestrus (10), 18.00 h. oestrus (6) and 18.00 h. dioestrus (8). Concentrations of LH-RF were determined in duplicate (where possible) aliquots of 100 µl, in Assays 22, 24, 27, 29, and 31.

The results of these experiments appear in Table 4.5 and Figure 4.3 and are summarised in Table 4.6. At each of the times of the oestrous cycle examined, stalk plasma volume collected, LH-RF concentration and LH-RF content increased during the period of stimulation. With respect to LH-RF content, these increases during stimulation were significant at all times examined ($p < 0.02$) except at 18.00 h dioestrus ($p < 0.1$). When the LH-RF contents of plasma collected prior to stimulation are compared, it appears that levels at 18.00 h. oestrus, and at 18.00 h dioestrus are markedly lower than those during the afternoon of pro-oestrus, and lower than those at 18.00 h. metoestrus. However, these differences are not significant. A similar picture appears when LH-RF contents of plasma collected during stimulation are compared, but again differences between groups are not significant.

C Animals subjected to two periods of electrical stimulation in the medial preoptic area, pituitary stalk blood collection lasting for two hours.

In a number of the experiments described in A and B, stalk blood collection was continued after the first two 30 min. periods for two further 30 min. periods, stimulation being again applied in the fourth 30 min. period. The LH-RF contents of blood collected in the four consecutive periods appear in Figure 4.4 for four male animals, and in Figure 4.5 for six female animals. It is apparent

TABLE 4.5

No.	Plasma Volume (ml)		LH-RF Concentration pg/ml		LH-RF Content pg/30 min.	
	A	B	A	B	A	B
DIOESTRUS 1800						
1	0.08	0.07	133.0	174.0	10.6	12.2
2	0.18	0.83	53.0	123.0	9.5	102.1
3	0.42	0.75	54.0	92.0	22.7	69.0
4	0.29	0.38	26.0	36.0	7.5	13.7
5	0.20	0.56	49.0	100.0	9.8	56.0
MEAN	0.23	0.52	63.0	105.0	12.0	50.6
± S.E.M.	0.06	0.14	18.0	22.4	2.7	17.1
PRO-OESTRUS 0900						
1	0.51	1.24	32.0	84.0	16.0	104.0
2	0.20	0.43	82.0	168.0	16.0	72.0
3	0.25	0.57	32.0	126.0	7.9	71.8
4	0.22	0.52	110.0	264.0	24.2	137.3
5	0.30	0.53	157.0	234.0	47.1	124.0
6	0.49	1.63	143.0	89.0	70.0	145.0
7	0.45	0.95	165.0	73.0	74.0	69.0
MEAN	0.35	0.84	103.0	148.2	36.5	103.3
± S.E.M.	0.05	0.17	21.2	28.8	10.3	12.4
PRO-OESTRUS 1300						
1	0.13	0.23	66.0	84.0	9.0	19.0
2	0.76	1.05	76.0	106.0	58.0	113.0
3	0.19	0.57	92.0	172.0	17.6	98.0
4	0.65	1.02	32.0	70.0	20.0	71.0
5	0.30	0.70	64.0	460.0	19.2	322.0

.../contd.

No.	Plasma Volume (ml)		LH-RF Concentration pg/ml		LH-RF Content pg/30 min.	
	A	B	A	B	A	B
PRO-OESTRUS 1300						
6	0.92	1.12	62.0	94.0	57.0	105.0
7	0.69	1.49	146.0	182.0	101.0	271.0
8	0.30	0.65	37.0	400.0	11.2	260.0
MEAN	0.49	0.85	71.9	196.0	36.6	157.4
± S.E.M.	0.10	0.14	12.6	53.3	11.5	39.1
PRO-OESTRUS 1800						
1	0.16	0.28	91.0	316.0	15.0	88.0
2	0.46	1.01	49.0	55.0	25.0	58.0
3	0.40	0.72	76.0	103.0	30.4	96.7 *
4	0.59	0.63	57.0	58.0	33.6	36.5
5	0.54	0.92	29.0	75.0	15.7	69.0
6	0.25	0.80	224.0	153.0	56.0	122.0
7	0.25	0.45	~~306.0~~ 428.0	394.0	107.0	177.0
8	0.54	0.87	46.0	94.0	25.0	82.0
9	0.26	0.71	50.0	50.0	13.0	36.0
10	0.25	0.40	140.0	182.0	35.0	72.8
MEAN	0.37	0.68	106.8	148.0	35.6	83.8
± S.E.M.	0.05	0.07	28.8	37.6	8.9	13.3
OESTRUS 1800						
1	0.38	0.38	27.0	108.0	10.0	41.0
2	0.34	0.41	64.0	196.0	21.8	80.4
3	0.18	0.63	47.0	89.0	8.5	56.0
4	0.28	0.37	29.0	340.0	8.1	125.8
5	0.45	0.44	15.0	57.0	6.8	47.1 **
6	0.36	0.94	26.0	48.0	9.4	45.5

* Collection 23 min only. Extrapolated to 30 min.

** " 16 " " " " " "

contd.

No.	Plasma Volume (ml)		LH-RF Concentration pg/ml		LH-RF Content pg/30 min.	
	A	B	A	B	A	B
OESTRUS 1800						
MEAN	0.33	0.53	34.6	139.7	10.8	65.9
± S.E.M.	0.04	0.25	7.2	45.5	2.3	13.3
METOESTRUS 1800						
1	0.49	0.66	135.0	123.0	66.2	81.2
2	0.28	0.51	46.0	145.0	12.9	74.0
3	0.58	0.75	38.0	96.0	22.0	72.0
4	0.29	0.35	43.0	135.0	12.5	47.3
5	0.66	1.10	36.0	114.0	24.0	125.4
6	0.25	0.60	53.0	173.0	13.0	104.0
7	0.25	0.95	64.0	128.0	16.0	122.0
8	0.20	0.40	37.0	424.0	7.4	169.6
MEAN	0.38	0.67	56.5	167.0	21.8	99.4
± S.E.M.	0.06	0.09	11.7	37.0	6.6	13.8

TABLE 4.5 Volumes, LH-RF concentrations and LH-RF contents of portal plasma collected from female animals bearing chronically implanted electrodes in the medial preoptic area. Two consecutive 30 min. collection periods; electrical stimulation in the second 30 min. period only.

A = 0 - 30 min.

B = 30 - 60 min. (Stimulation period).

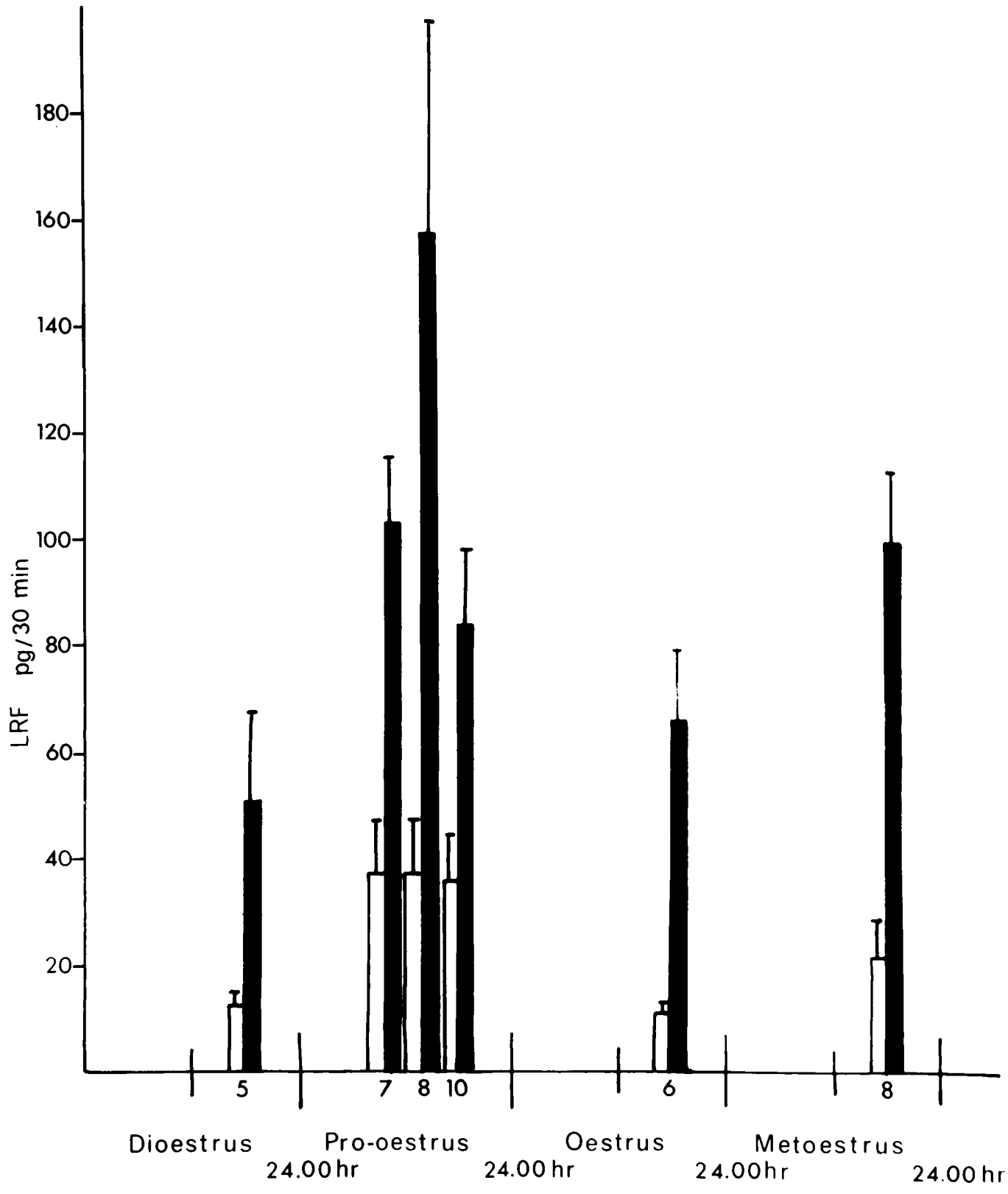


Figure 4.3 Histogram showing the mean content of LH-RF in pituitary stalk blood collected before (open bars) and during (closed bars) the application of electrical stimulation to the medial preoptic area at various times during the oestrous cycle. Vertical lines at tops of bars indicate ± S.E.M., while figures at bases of bars indicate the numbers of animals in each group.

	Volume (ml)		LH-RF Concentration (pg/ml)		LH-RF Content (pg/30 min.)	
	A	B	A	B	A	B
DIOESTRUS 1800 n = 5 (MEAN ± S.E.M.)	0.23 ± 0.06	0.52 ± 0.14	63.0 ± 18.0	105.0 ± 22.4	12.0 ± 2.7	50.6 ± 17.1 p < 0.1
PRO-OESTRUS 0900 n = 7	0.35 ± 0.05	0.84 ± 0.17	103.0 ± 21.2	148.2 ± 28.8	36.5 ± 10.3	103.3 ± 12.4 p < 0.01
PRO-OESTRUS 1300 n = 8	0.49 ± 0.10	0.85 ± 0.14	71.9 ± 12.6	196.0 ± 53.3	36.6 ± 11.5	157.4 ± 39.1 p < 0.02
PRO-OESTRUS 1800 n = 10	0.37 ± 0.05	0.68 ± 0.07	106.8 ± 28.8	148.0 ± 37.6	35.6 ± 8.9	83.8 ± 13.3 p < 0.01
OESTRUS 1800 n = 6	0.33 ± 0.04	0.53 ± 0.25	34.6 ± 7.2	139.7 ± 45.5	10.8 ± 2.3	65.9 ± 13.3 p < 0.02
METOESTRUS 1800 n = 8	0.38 ± 0.06	0.67 ± 0.09	56.5 ± 11.7	167.0 ± 37.0	21.8 ± 6.6	99.4 ± 13.8 p < 0.01

TABLE 4.6 Summary. Volumes, LH-RF concentrations and LH-RF contents of portal plasma collected from female animals bearing chronically implanted electrodes in the medial preoptic area. Two consecutive 30 min. collection periods; electrical stimulation in the second 30 min. period only.

A = 0 - 30 min.

B = 30 - 60 min. (Stimulation period)

Paired t-test.

Figure 4.4 Histogram showing LH-RF content of pituitary stalk plasma collected over four consecutive 30 min. periods from four male animals (see text).

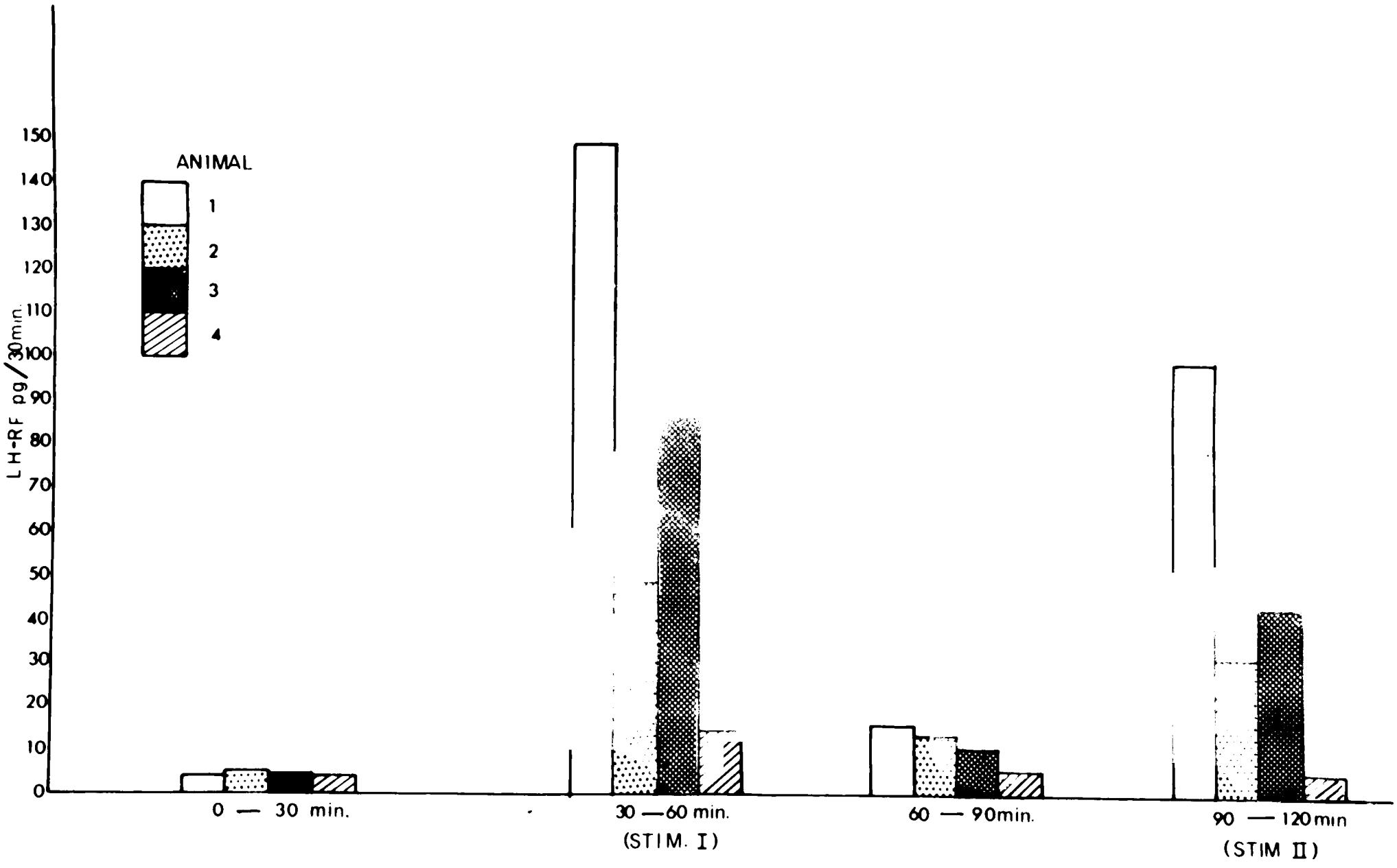
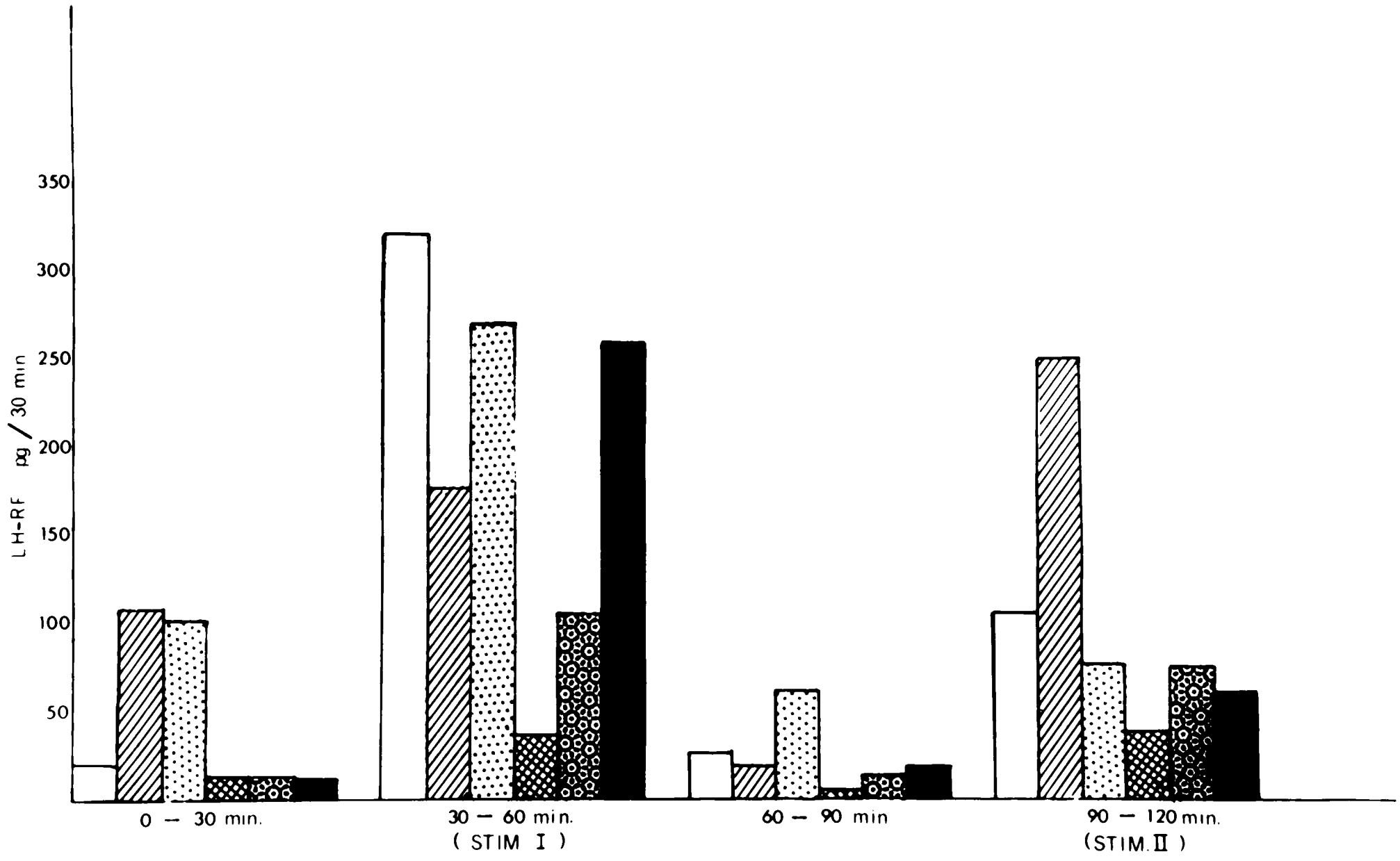


Figure 4.5 Histogram showing LH-RF content of pituitary stalk plasma collected over four consecutive 30 min. periods from 6 female animals (see text).



that in three of four males, and in all of the females, stimulation for a second period leads to a second increase in LH-RF content of stalk plasma, although the increases are generally not as marked as they are in the first period of stimulation.

D Female animals, at dioestrus, and after ovariectomy.

Burger, Fink & Lee (1972) and Ben-Jonathan, Mical & Porter (1973) have observed by means of in-vivo and in-vitro bioassays respectively that there are increased levels of LH-RF in portal plasma of ovariectomised rats. These observations, together with those of Gay and Midgley (1969) and Yamamoto, Diebel & Bogdanove (1970) that LH release from the anterior pituitary is increased after ovariectomy, prompted the examination of pituitary stalk plasma for LH-RF content after ovariectomy.

Present Experiments. Stalk blood was collected for one hour periods from 7 female rats on the afternoon of dioestrus (13.00 h.) and from 8 rats ovariectomised at least four weeks previously. Concentrations of LH-RF were determined in Assay 22 (50% intercept, 67 pg/ml).

The results of these experiments appear in Table 4.7. There is no significant difference between stalk plasma volumes or content of LH-RF in the two groups, but the concentration of LH-RF in dioestrous stalk plasma (36.9 $\pm$ 4.5 pg/ml), was significantly greater than that in ovariectomised stalk plasma (22.4 $\pm$ 3.3 pg/ml), $p < 0.01$.

NO.	DIOESTRUS CONTROL			NO.	OVARIECTOMISED		
	Plasma Volume (ml)	LH-RF Concentration pg/ml	LH-RF Content pg/hr.		Plasma Volume (ml)	LH-RF Concentration pg/ml	LH-RF Content pg/hr.
1	0.30	52.0	15.6	1	0.60	19.0	11.4
2	1.00	19.5	19.5	2	0.90	23.0	20.7
3	0.80	25.0	10.0	3	0.40	31.0	12.4
4	0.40	33.9	15.2	4	0.90	24.0	21.6
5	0.60	37.4	22.4	5	0.80	72.0	17.6
6	0.60	42.4	25.4	6	0.50	14.5	7.3
7	0.80	48.4	19.4	7	1.10	27.0	29.7
				8	0.50	19.0	9.5
MEAN	0.53	36.9	18.2		0.70	22.4	16.3
± S.E.M.	±0.09	4.5	1.9		0.10	3.3	7.1

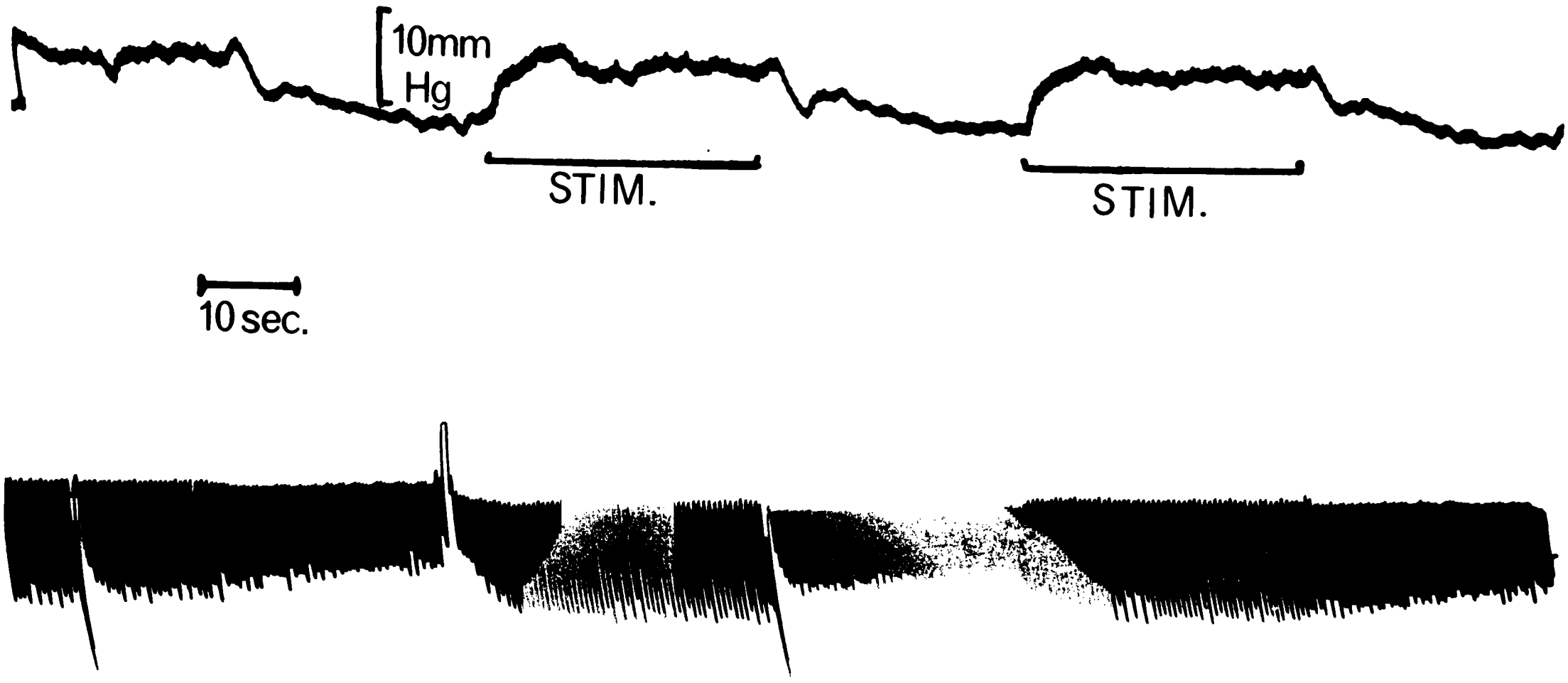
TABLE 4.7 Volumes, LH-RF concentrations and LH-RF contents of portal plasma collected over a one hour period from chronically ovariectomised rats, and from intact rats at dioestrus.

E General cardio-respiratory effects of stimulation in the medial preoptic area.

Urethane anaesthesia as described proved adequate for periods of electrical stimulation and pituitary stalk blood collection of two hours. With a.c. stimulation at higher pulse amplitudes and durations, visible effects of stimulation appeared to be vibrissal tremor, contraction of the levator palpebrae, and occasionally laryngeal stridor. Because of the effect that cardiorespiratory changes might have on stalk blood flow, attempts were made to characterise these responses.

In experiments on five animals, it was found that biphasic stimulation via twin unipolar platinum electrodes at 60 Hz, pulse amplitude 0.5 mA, pulse duration 1 msec, 30 sec. on/off, led to increases in blood pressure (B.P.) of 8-20 mm Hg from basal levels of 130 mm Hg. The heart rate fell from basal levels of 380-420/min. to 280-320/min., and respiration rate fell from basal levels of 150-160/min. to 100-110/min., accompanied by an increase in respiratory depth of 50-60%. A representative tracing of B.P. and respiration appears in Figure 4.6. B.P. rises as stimulation begins, achieving maximal levels after 8-10 sec., and remains steady until 5 sec. after the stimulation period ends after which it declines over a period of 15 sec. to the baseline. The respiratory response to stimulation begins and ends almost simultaneously with the

Figure 4.6 Photograph of pen tracings of records of B.P. and heart rate (upper trace) and respiration rate and depth (lower trace) in rats electrically stimulated in the medial preoptic area (see text).



switching on and off of stimulation.

These effects of stimulation of B.P. were not primarily (if at all) due to discharge of vasopressin in these animals, since the B.P. change produced by injection of vasopressin at a dose of 40 mu/kg produced an increase in B.P. of 20 mm Hg which slowly declined over a period of 5 min. The responses in heart rate, B.P., respiration rate, and respiration depth were depressed only slightly by administration of either atropine, propanolol, phentolamine or phenoxybenzamine.

4.6. DISCUSSION

It is apparent that extracts of pituitary stalk plasma cause the release of both LH and FSH from anterior pituitary glands incubated in-vitro. The calculated potencies of stalk plasma in terms of the decapeptide standard differ widely, particularly when the male and female assay systems are compared. On only one occasion was it possible to show that the dose-response curves for synthetic LH-RF and for stalk plasma were parallel. This in itself is an advance on previous investigations in which dose response curves have not been obtained, but it is hardly conclusive evidence that gonadotrophin releasing substance in portal blood is identical to the synthetic LH-RF preparation. The female assay system is less sensitive to stalk plasma ultrafiltrate than is the male system. The doses of stalk plasma

ultrafiltrate, and the conditions of collection of stalk blood are comparable in assays 2 and 3 (male system) and 8, 11, and 14 (female system), and yet potencies of stalk plasma ultrafiltrate for both LH and FSH release in the male assay system are generally much greater than they are in the female assay system.

In assay 21, the stalk plasma ultrafiltrate was collected not only under conditions in which the stimulation parameters used were those that are optimal for discharge of LH in animals with intact hypothalamo-hypophysial axes, but also in two periods, in the first of which no stimulation was applied. Calculation of potencies reveals that rather more gonadotrophin releasing substance was present in stalk blood during the period of stimulation. These results confirm the observations of Burger et al. (1972) who injected ultrafiltrates of pituitary stalk plasma collected from male animals prior to or during stimulation of the medial preoptic area, into OOP assay rats. The mean of increments in serum LH in the assay animals treated with ultrafiltrate from the stimulation period was greater than that in animals treated with ultrafiltrate from the non-stimulation period.

Failure to obtain dose-response curves in the female system may be due to the fact that releasing activity in pituitary stalk plasma ultrafiltrates was too low. Collection of sufficient stalk plasma to provide for two doses of ultrafiltrate with four assay

flasks per dose is a laborious task if each flask at the higher dose is to receive an amount of ultrafiltrate equivalent to even 40 min. of stalk blood collection.

The fact that stalk plasma ultrafiltrate causes release of both LH and FSH in these assay systems is of considerable importance. Initially it was thought that LH-releasing and FSH-releasing activities were the properties of two different substances (Schally, Arimura, Bowers, Kastin, Sawano & Redding, 1968). It was then found that both highly purified LH-RF of porcine origin and the synthetic preparation of LH-RF stimulated both LH and FSH release in men and women and in a variety of animal species, and it was proposed that one hypothalamic hormone was responsible for stimulating the release of both LH and FSH from the anterior pituitary gland (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White, 1971). The present studies tend to support this view in adding the important observation that portal blood of rats contains material capable of releasing both LH and FSH from the anterior pituitary gland over similar time periods. It should be remembered however, that the failure to obtain parallel dose-response curves for synthetic LH-RF and for stalk plasma ultrafiltrate in more than one of six assays raises doubts as to whether the gonadotrophin releasing substance measured in the portal plasma ultrafiltrate is identical to synthetic LH-RF.

Measurements made by radioimmunoassay suggest that LH-RF is measurable in pituitary stalk plasma collected under a variety of experimental conditions. The evidence that in male animals stimulation of the medial preoptic area leads to rises in the LH-RF content of pituitary stalk blood, compares satisfactorily with the evidence presented in Chapter 2 that rises in peripheral LH concentration also follow stimulation of the medial preoptic area. The important index of LH-RF secretion into hypophysial portal blood is the content, not the concentration of LH-RF. Because the blood collected by Worthington's method is not solely portal blood, the concentration of LH-RF in the blood collected is not a true measure of LH-RF secretion. Total content of LH-RF is a better measure, as contributions to the blood collection from other sites have minimal effects on the amount of LH-RF present, since levels of LH-RF in systemic plasma are low or undetectable in the LH-RF radioimmunoassay.

The results of determinations of LH-RF content in pituitary stalk plasma prior to and during electrical stimulation in the medial preoptic area of female rats at various times in the oestrous cycle reveal that as in the male, contents of LH-RF in stalk plasma are considerably higher in the stimulation period than in the pre-stimulation period. Differences between LH-RF contents of pre-stimulation and stimulation samples were significant at all times

examined except at 18.00 h. dioestrus when the relative degree of variation in the LH-RF contents of stimulation samples was greater than at the other times in the cycle. While there was no significant difference between LH-RF contents in the pre-stimulation samples collected at various times in the cycle, the increased levels of LH-RF at pro-oestrus and at metoestrus may correspond respectively to the pre-ovulatory surge of peripheral plasma LH at pro-oestrus and the slight rise in peripheral plasma LH at metoestrus. It may be that if LH-RF secretion occurs in episodic bursts, then the length of time over which collections of stalk blood are made obscures differences which may exist during the oestrous cycle. Clearly it would be ideal if measurements of LH-RF content could be made on samples collected over shorter time periods. Both the variation in stalk blood flow rates between animals, and the difficulty in handling and assaying very small quantities of plasma would make this approach difficult. Again the true picture may be obscured by the anaesthesia. It has been pointed out previously (see Chapter 2) that urethane can block ovulation in the rat, and it may well have a depressive effect on discharge of LH-RF from the median eminence.

A comparable study, using an OAAD assay for measuring LH-RF activity has been made by Harris and Ruf (1970), who stimulated female rats in the median eminence with twin unipolar platinum electrodes for a one hour period (parameters, 30 Hz, biphasic pulses,

pulse amplitude 0.5 mA, pulse duration 1 msec., 30 sec on/off). They found that electrical stimulation of the median eminence during the collection of stalk blood increased the OAAD activity of stalk blood at metoestrus and proestrus, but not at oestrus.

Fink and Aiyer (1974), in commenting on their studies both of hypothalamo-pituitary sensitivity to electrical stimulation of the medial preoptic area in the female and of pituitary sensitivity to injections of synthetic LH-RF, suggest that while their results "do not exclude a change in sensitivity of the neural component of the hypothalamo-pituitary axis during the oestrous cycle, they do indicate that the timing and magnitude of changes in sensitivity of the anterior pituitary gland to both endogenous and exogenous LH-RF are similar". They raise the possibility that a marked increase in the secretion of LH-RF during the afternoon of pro-oestrus may not be necessary for the occurrence of the surge of LH and FSH, for conceivably the surge could depend on a change in sensitivity of pituitary gonadotrophs to constant or only slightly increased levels of LH-RF in hypophysial portal blood.

The results of the present studies confirm the wisdom of that view. There is clearly no marked rise in LH-RF secretion into hypophysial portal blood observable during pro-oestrus, although, as has been said, anaesthesia may depress differences in secretion rate.

If there were an increase in the sensitivity of the hypothalamo-median eminence-portal vessel axis to electrical stimulation at the time of pro-oestrus, then it might be expected that the increment in LH-RF content during stimulation would be greatest at 18.00 h. of pro-oestrus when the sensitivity of the entire hypothalamo-pituitary axis to stimulation under Nembutal anaesthesia is maximal (Fink & Aiyer, 1974). In the present studies the greatest increment in LH-RF content during stimulation under Urethane anaesthesia occurs however at 13.00 h. of pro-oestrus. Subtle changes in sensitivity may of course be overridden by the electrical stimulation parameters employed; the different anaesthetics employed in the two studies add to the difficulties in making direct comparisons.

Failure to observe increased levels of immunoassayable LH-RF in pituitary stalk plasma from ovariectomised rats as compared with dioestrous controls is in conflict with the results of Fink & Harris (1970), Burger et al. (1972), and Ben-Jonathan et al. (1973). Because LH release from the anterior pituitary is increased after ovariectomy (Gay & Midgley, 1969; Yamamoto et al., 1970) it is conceivable that the chronic absence of gonadal steroids results pari-passu in an increased secretion of LH-RF. Again, the failure in the present studies may be due to the fact that pituitary stalk blood was collected for insufficient time periods to make differences

in secretion rates readily observable.

Much has been made previously (see Chapter 2) of the lesioning effects both of insertion of electrodes, and of electrical stimulation. It is encouraging in this respect that in the experiments involving chronic implantation of electrodes into the medial preoptic area of female rats, normal oestrous cycles (as assessed by vaginal smearing) still occur. In some animals, a period of several days of constant dioestrus followed implantation, but in almost all cases there was eventual resumption of normal cycles. The results of the experiments in which two periods of stimulation occurred add strength to the belief that the stimulation parameters employed for a.c. stimulation are such as to interfere minimally with function of the ventral diencephalon. In nine of ten animals a second period of stimulation resulted in a second rise in LH-RF content of pituitary stalk blood. Examination of Figures 4.4 and 4.5 reveals that the LH-RF content of blood collected in the third 30 min. period (no stimulation) differ little from the LH-RF contents of blood collected in the first 30 min. period. This suggests that the electrical stimulation and the secretion of LH-RF are temporally closely associated.

The studies of cardiorespiratory responses to medial preoptic area stimulation reveal that stimulation raises blood pressure. No information is available on the resistance of the primary capillary plexus or of the portal vessels in the pituitary stalk. In its

absence, it is presumed that increased volumes of pituitary stalk plasma collected during stimulation are due to the raised blood pressure. If the increases in LH-RF concentration and content observed during stimulation were to be explained on the basis of increased flow rates alone, then one might expect that the content of LH-RF over a certain time period would vary directly with volume of plasma collected. This is clearly not so. While volumes of plasma collected may more than double during stimulation, the content of LH-RF generally more than trebles, and may increase as much as six-fold.

Mere recording of blood pressure, heart rate, and respiration rate can give little information regarding the detailed effects of diencephalic electrical stimulation (Bard, 1960). The responses described in the present studies seem to be primarily centrally mediated, since neither the parasympatholytic drug atropine, nor the sympathetic α and β blockers were capable of interfering markedly with the responses.

The results presented in this chapter demonstrate that it is possible to measure endogenous LH-RF in pituitary stalk plasma by both in-vitro bioassay systems and by RIA. Only in one circumstance, namely during electrical stimulation of the medial preoptic area of male rats have measurements been made using both assay methods for purposes of comparison. Such a comparison raises some problems. In the first place, the pituitary stalk blood collection conditions

differ in part, the collections used for bioassay (except in the case of bioassay No. 21) being made over a period of one hour, those for RIA over a period of a half-hour. Secondly, the biphasic pulses used for stimulation had in the case of collections for bioassay (except in the case of bioassay No. 21) durations of 2 msec; those for RIA had durations of 1 msec. In the case of bioassay No. 21, the circumstances of collection were identical with those for RIA.

The effect of the differences mentioned is such that pituitary stalk collections for bioassay are likely to contain rather less LH-RF than those for RIA. On comparison of the results however, an enormous disparity is evident. By RIA, the LH-RF content of portal blood collected over a 30 min. period of stimulation is 84 pg. By bioassay, the LH-RF content of similar stalk blood ranges from 1.1 to 30 ng for LH release and from 0.3 to 16 ng for FSH release (discarding bioassay No. 2 in which no controls are available). There is therefore approximately a 4-360 fold difference between the results of the two assay systems. This enormous disparity may well be an underestimate when the question of recovery of LH-RF in extraction and ultrafiltration procedures is considered.

There are several possible explanations for the disparity. First, it has been observed before that bioassay and RIA estimates of hormone concentrations are often in poor agreement. Burger et al.

(1972) for example, have compared results of the OAAD bioassay with those of the RIA for LH as reported by other workers in terms of NIH-LH-SI and find that the mean ratio OAAD LH : RIA LH is 3.4.

Secondly, the in-vitro bioassay results may be artefactual. Burger et al. (1972) using the OOP rat for in-vivo bioassay found that mean increments in serum LH after the injection of ultrafiltrates of pituitary stalk or systemic plasma obtained from ovariectomised rats were similar. A possible explanation for this finding is that the extraction/ultrafiltration procedure may produce material other than LH-RF which is capable of release of LH in-vivo, and perhaps in the present studies in-vitro.

Thirdly, the RIA results may be artefactual. The evidence presented in Chapter 3 regarding the parallelism between inhibition curves for synthetic LH-RF (standard) and inhibition curves for pituitary stalk plasma provides good assurance that what is measured in the LH-RF RIA is identical to the synthetic material. It may be however, that the procedures used in the original extraction from porcine and ovine hypothalami of the LH-RF subsequently characterised and synthesised as a decapeptide, have resulted in extraction of a substance which bears little relation to the physiological gonadotrophin releasing substance to be found in the median eminence and in hypophysial portal blood in life. In life it may be in the form of a pro-hormone, or it may be bound to a protein both within the

median eminence and in portal blood. In this respect the existence of specific binding proteins, neurophysins, for the posterior pituitary octapeptides oxytocin and vasopressin (Frieson & Astwood, 1967; Hope, 1970) is of relevance. In the extraction procedures "free" LH-RF may be liberated from such a binding protein. The gonadotrophin releasing activity of ultrafiltrates of stack plasma may therefore be greatly enhanced in both in-vivo and in-vitro bioassays. Nett et al. (1974) in considering their determinations of LH-RF concentration in systemic blood of the ewe, propose that "two LH releasing factors may be present in the hypothalamus. One, identical with the synthetic decapeptide to which the antibodies were produced, which is responsible for the tonic release of LH . . . the other, not yet identified, may be the LRF responsible for the climactic preovulatory surges of LH".

CHAPTER 5

CONCLUSIONS

5.1 INTRODUCTION

Electrical stimulation techniques have been used in studies on the hypothalamo -hypophysial axis of the rat. The establishment of both in-vitro bioassays and a radioimmunoassay for luteinising hormone-releasing factor (LH-RF) has allowed the measurement of the LH-RF content of a variety of materials including pituitary stalk plasma collected by the method of Worthington (1966) under a variety of experimental conditions.

5.2. Electrical Stimulation in the Diencephalon.

Both alternating current (a.c.) and direct current (d.c.) stimulation of the medial preoptic area of male rats lead to significant rises in peripheral plasma luteinising hormone concentration. With respect to a.c. stimulation the most effective parameters of stimulation are frequency 60 Hz, pulse amplitude 0.5 mA and pulse duration 1 msec, the stimulus being in the form of biphasic square waves. The response of the neural elements stimulated is not directly related to either pulse coulombage or to total coulombage of current passed, as increases in pulse and total coulombage beyond the limits set by the most effective parameters leads to smaller increases in the peripheral plasma LH concentration than those experienced with stimulation with optimal parameters.

D.c. stimulation of the medial preoptic area in male rats is

effective when steel electrodes are used, but not when platinum electrodes are used. In both a.c. and d.c. stimulation with steel electrodes, ferric ions are deposited in the region of electrode terminations. The effect of steel electrode a.c. and d.c. stimulation is prolonged when compared with the effect of a.c. stimulation with platinum electrodes. It appears that this prolongation of effect is due to the deposition of ferric ions. The importance of differentiating between short- and long-term effects of electrical stimulation, and between stimulation and lesion effects is stressed, and complete reporting of lesion and/or stimulation parameters including electrode materials is urged.

The fact that electrical stimulation of the medial preoptic area is followed by a rise in the concentration of peripheral plasma LH, suggests that this region of the brain is an important component of the neural mechanism which controls the secretion of LH in the male as well as in the female rat.

5.3. Assays for LH-RF

The discovery and synthesis of LH-RF has allowed the development of quantitative in-vitro bioassays for LH-RF, and the establishment in this laboratory of a radioimmunoassay for LH-RF.

In in-vitro bioassays, both male rat anterior pituitary glands

from ovariectomised oestrogen/progesterone pre-treated female rats release significant amounts of both LH and FSH into incubation medium when synthetic LH-RF is added. Satisfactory dose-response relationships have been obtained in both assay systems, but the female system is preferred as only one pituitary per flask is needed, whereas 3-4 pituitaries per flask are needed in the male system. There is considerable between assay variation in both assay systems, but indices of precision are satisfactory. Concentrations of K^+, adrenaline, noradrenaline, dopamine, vasopressin and oxytocin considerably in excess of those that might be expected to occur in extracts of hypophysial portal blood do not cause release of either LH or FSH from female anterior pituitary glands incubated in-vitro.

Full activity of synthetic LH-RF in in-vitro bioassay for release both of LH and FSH from anterior pituitary glands is dependent on the presence of the entire chain length of LH-RF, as removal of the C-terminal amino acid results in depression of LH and FSH releasing activity by 85-95%. A number of other synthetic analogues of LH-RF have minimal if any LH and FSH releasing activity.

Radioimmunoassay for LH-RF using synthetic LH-RF as the standard preparation has been established in this laboratory using an antiserum (developed by Dr. G. Niswender, Colorado State University, U.S.A.) capable of avid and specific binding of both radioiodinated and unlabelled synthetic LH-RF. It has been demonstrated that it is

appropriate to use this assay for the measurement of LH-RF in a number of natural materials containing added synthetic LH-RF and in natural materials containing endogenous LH-RF. Evidence regarding the specificity, sensitivity, and stability of the assay is presented.

5.4. Studies on Hypophysial Portal Blood.

Ultrafiltrates of pituitary stalk plasma collected during medial preoptic area electrical stimulation in male rats are capable of causing release of both LH and FSH from both male and female anterior pituitary glands incubated in-vitro. In only one of six in-vitro bioassays was it possible to show that dose-response curves for standard synthetic LH-RF and for pituitary stalk ultrafiltrate were parallel. Calculation of potency of pituitary stalk ultrafiltrate made either with a single dose of ultrafiltrate (five assays) or with two doses of ultrafiltrate (one assay) reveal considerable variation both in terms of LH release, and in terms of FSH release.

Measurement by RIA of LH-RF content of pituitary stalk plasma collected during medial preoptic area electrical stimulation in male rats, and in female rats at various times throughout the oestrous cycle reveal that such stimulation causes release of significantly increased amounts of LH-RF when compared with the amounts released in the period prior to stimulation. The increase in peripheral plasma LH concentration consequent upon stimulation of the medial preoptic area in both male and

female rats (with intact hypothalamo-hypophysial axes) can therefore be explained on the basis of the observed discharge of LH-RF into the hypophysial portal vessels.

In experiments which have a bearing on the studies of parameters of stimulation in the diencephalon, it was found that a second period of electrical stimulation following the first period after an interval of 30 min. was associated in nine of ten animals with a second rise in LH-RF content. This suggests first that the optimal parameters of stimulation used do not exhaust the preoptic area-median eminence axis of LH-RF; secondly that the electrical stimulation does not irretrievably damage the preoptic area neural elements that respond to such stimulation; and thirdly that medial preoptic area stimulation and secretion of LH-RF by the median eminence are temporally associated.

Measurement by RIA of LH-RF contents of pituitary stalk plasma collected from ovariectomised rats reveals that under the conditions of the present studies there is no increase in LH-RF content when comparison is made with intact dioestrous controls. The conflict between this finding and those of previous reports that bioassayable LH-RF is present in increased amounts in portal blood of ovariectomised animals, may be due to the fact that collections of pituitary stalk plasma were made over insufficiently long periods for differences to become evident.

While medial preoptic area electrical stimulation results in elevations of blood pressure and increases in pituitary stalk blood

flow rates, the elevations in stalk plasma LH-RF content observed during stimulation cannot be accounted for on this basis.

Major discrepancies between RIA and in-vitro bioassay measurements of LH-RF in hypophysial stalk plasma have been encountered. The possibilities that these discrepancies are due to artefacts in either form of assay, or to differences between synthetic LH-RF as extracted, and characterised from hypothalamic extracts, and the "physiological" form of endogenous LH-RF are considered.

A P P E N D I C E S

APPENDIX 2.1

Dixons 41 B Rat Diet.

Ingredients	Parts per Ton
Wheat	47
Sussex ground oats	40
Pure white fishmeal (66 % protein)	8
Unextracted dried yeast	1
Dried skim milk	3
Salt	1
Molasses	5

APPENDIX 2.2

Worksheet for Duncans Multiple Range Test

applied to data in frequency variation experiment (see Section 2.6)

At 40 min. ANALYSIS OF VARIANCE

Frequency (Hz)	No. of obs. (n)	Totals (T)	Means (x)
0	5	11.72	2.34
15	4	17.84	4.46
30	5	31.00	6.20
60	4	53.64	13.41
90	4	26.84	6.71
120	4	25.08	6.27
	N = 26	$\sum T = 166.12$	$\bar{x} = 6.38$

Sum of squares about the mean $\bar{x}$ $= \sum x^2 - \frac{(\sum x)^2}{N}$

$= 1619.20 - 1061.37$

$= 557.83$

Sum of squares between means $= \sum \left(\frac{T^2}{n}\right) - \frac{(\sum x)^2}{N}$

$= 1355.88 - 1061.37$

$= 294.51$

APPENDIX 2.2(cont.)

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares
Between varieties	294.51	5	58.90
Residual	263.32	20	12.53
		25	

Standard Error of a varietal mean $Sm = \frac{12.53}{5} = 1.58$

Multiple Range Test

5 % Level test

(a) Shortest Significant Ranges (Rp)

p:	2	3	4	5	6
Rp:	4.66	4.89	5.04	5.14	5.22

(b) Results

Frequencies	0	15	30	120	90	60
Means	2.34	4.46	6.20	6.27	6.71	13.41

Then testing	Significance
60 - 0 = 11.07	+
60 - 15 = 8.95	+
60 - 30 = 7.21	+
60 - 120 = 7.14	+
60 - 90 = 6.70	+
90 - 0 = 4.37	NS
90 - 15 = 2.15	NS
etc.	NS

APPENDIX 2.2(cont.)

1 % Level test

(a) Shortest Significant Ranges (Rp)

p:	2	3	4	5	6
Rp:	6.36	6.63	6.81	6.94	7.04

(b) Results

Frequencies	0	15	30	120	90	60
Means	2.34	4.46	6.20	6.27	8.71	13.41

Then testing

		Significance
60 - 0	= 11.07	+
60 - 15	= 8.95	+
60 - 30	= 7.21	+
60 - 120	= 7.14	+
60 - 90	= 6.70	+
90 - 0	= 4.37	NS
90 - 15	= 2.15	NS
etc.		NS

APPENDIX 2.3

FSH Responses to Stimulation in the Preoptic Area

The following six Figures (A1 - A6) present results of stimulation experiments in terms of peripheral FSH responses referred to in Section 2.12. All determinations were carried out on single samples of 100 µl.

Figure A.1 Peripheral plasma FSH concentrations prior to, during and after medial preoptic area a.c. stimulation at various frequencies (Platinum electrodes).

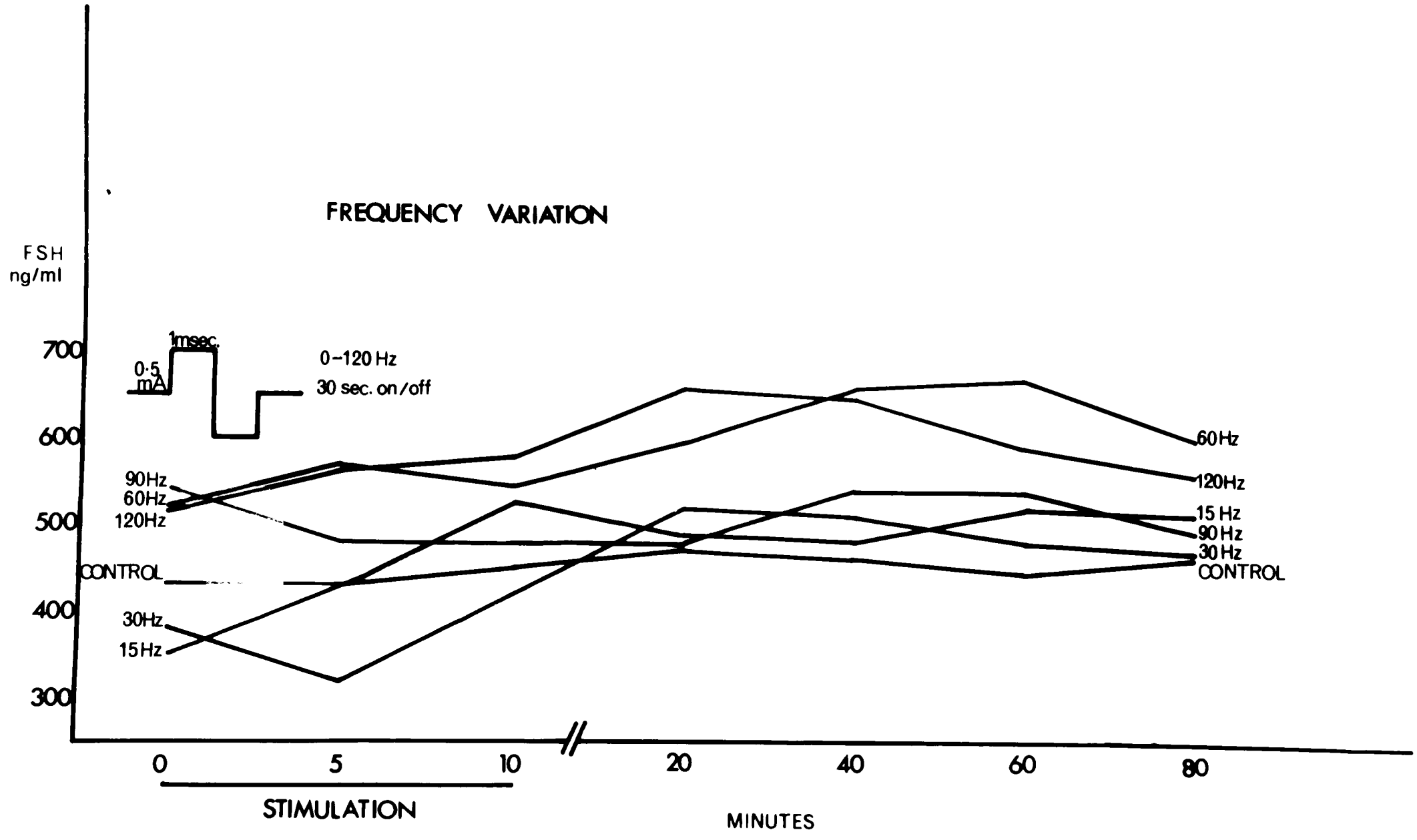


Figure A.2 Peripheral plasma FSH concentrations prior to, during and after medial preoptic area a.c. stimulation at various pulse amplitudes (Platinum electrodes).

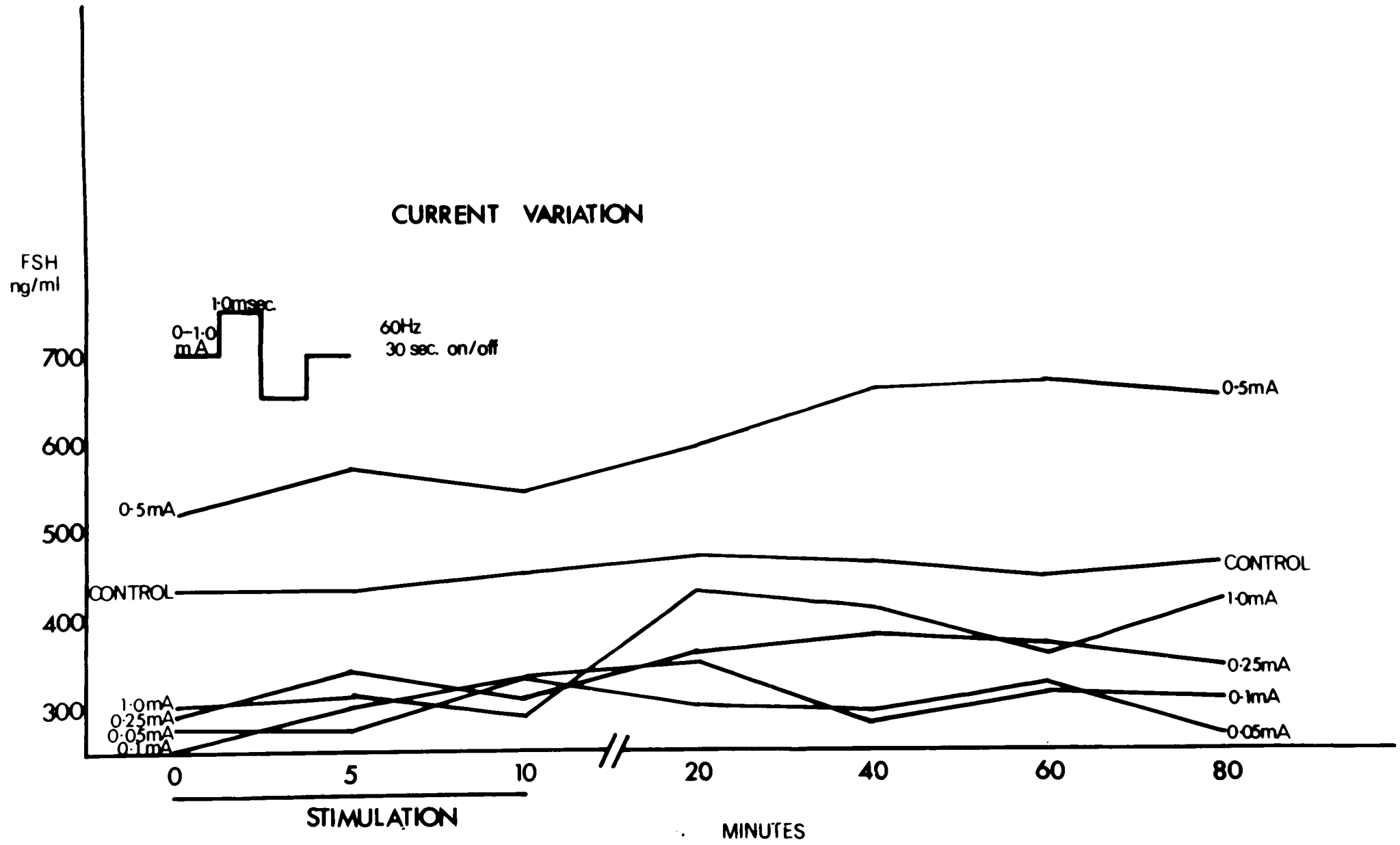


Figure A.3 Peripheral plasma FSH concentrations prior to, during and after medial preoptic area a.c. stimulation at various pulse durations (Platinum electrodes).

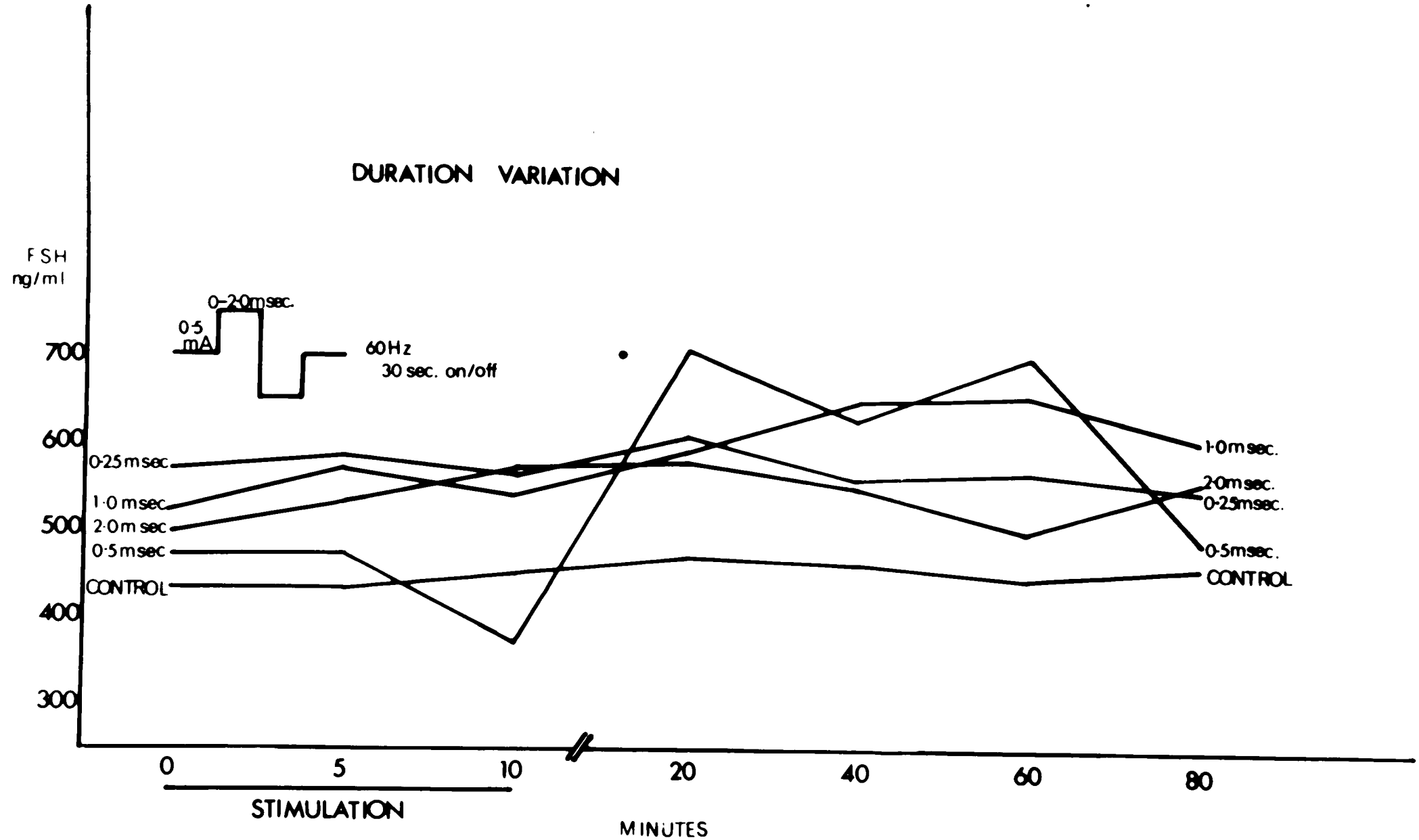


Figure A.4 Peripheral plasma FSH concentrations prior to, during and after medial preoptic area a.c. stimulation with either platinum or steel electrodes.

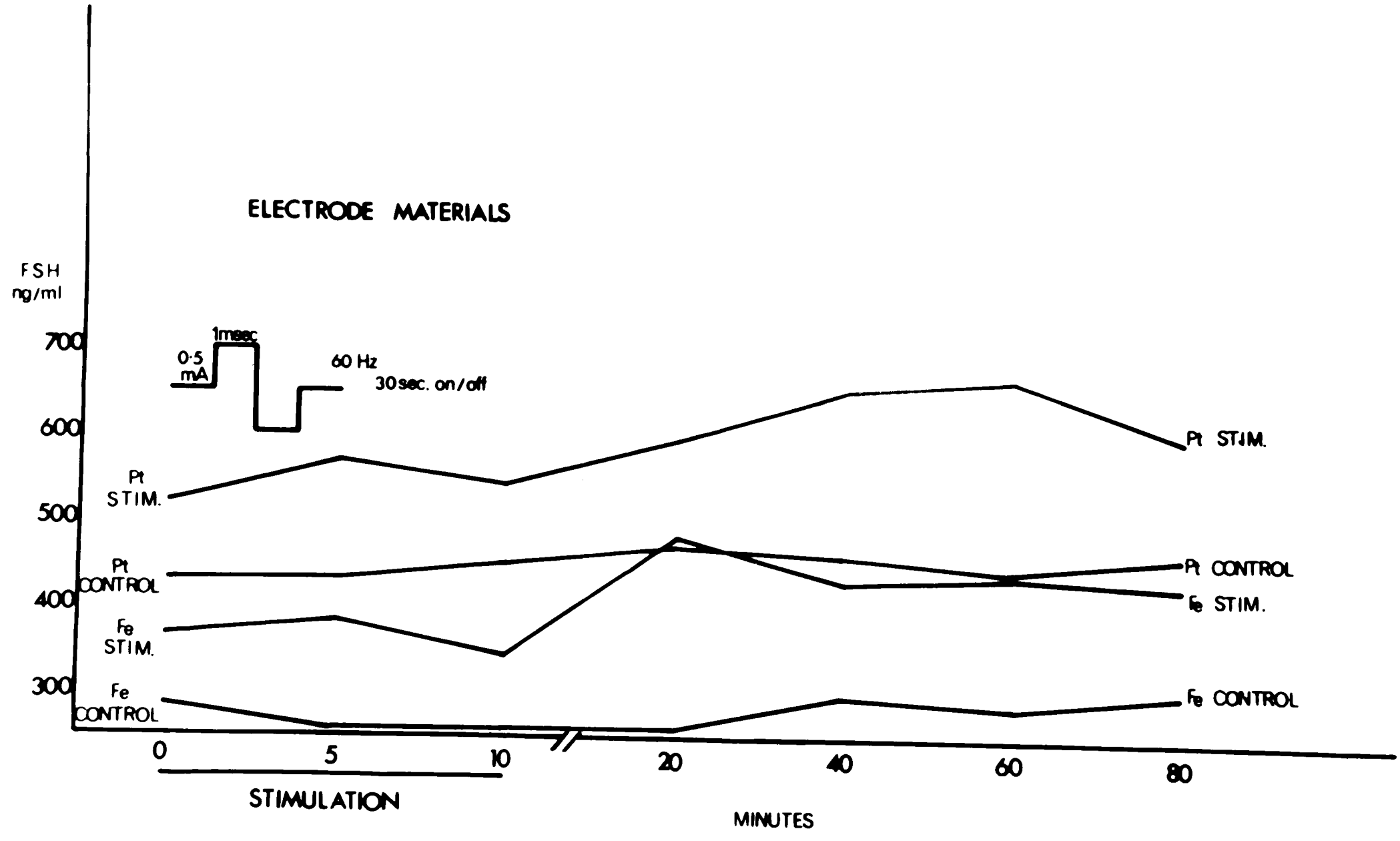


Figure A.5 Peripheral plasma FSH concentrations prior to, during and after medial preoptic area d.c. stimulation with either platinum or steel electrodes.

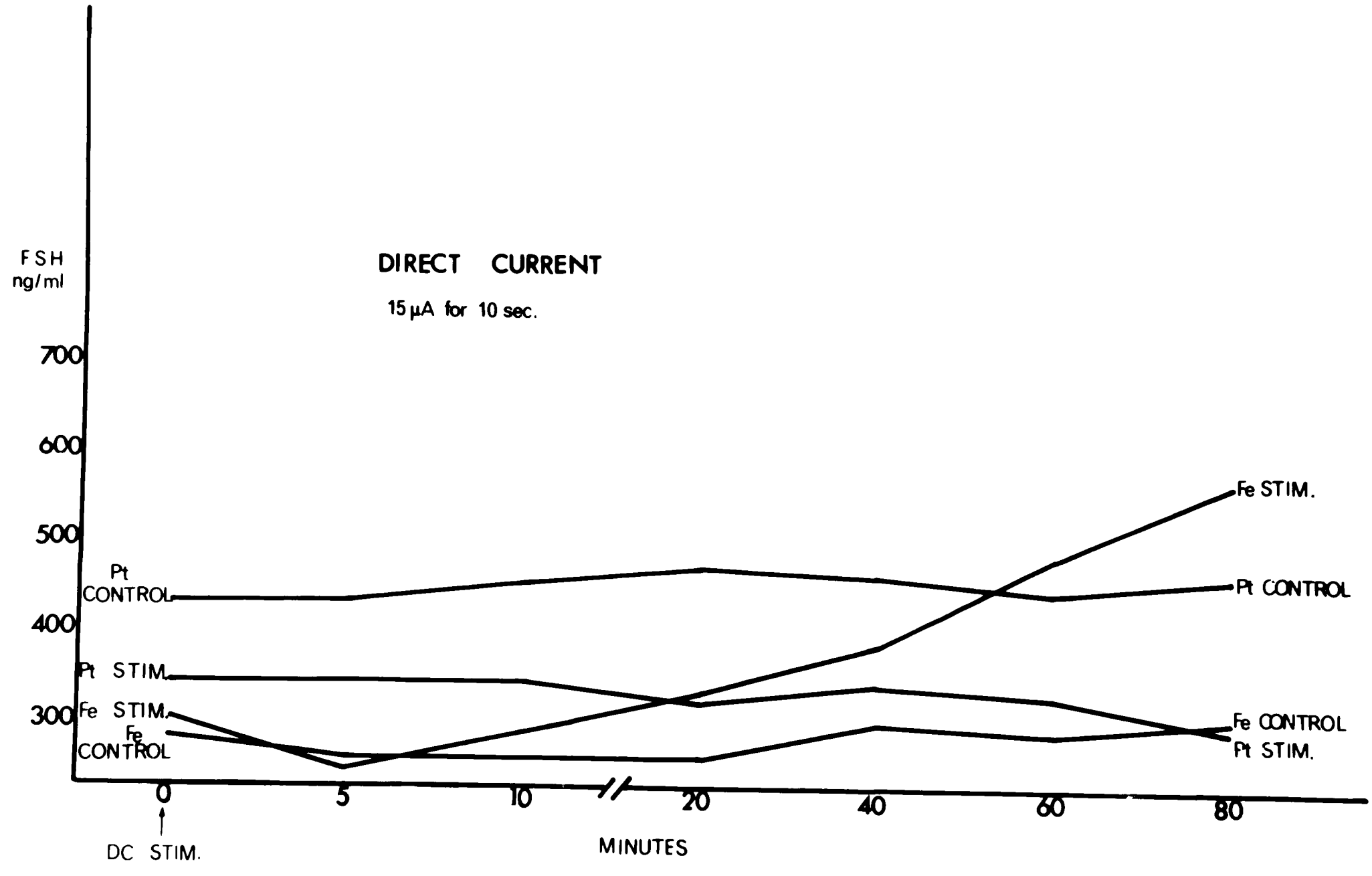
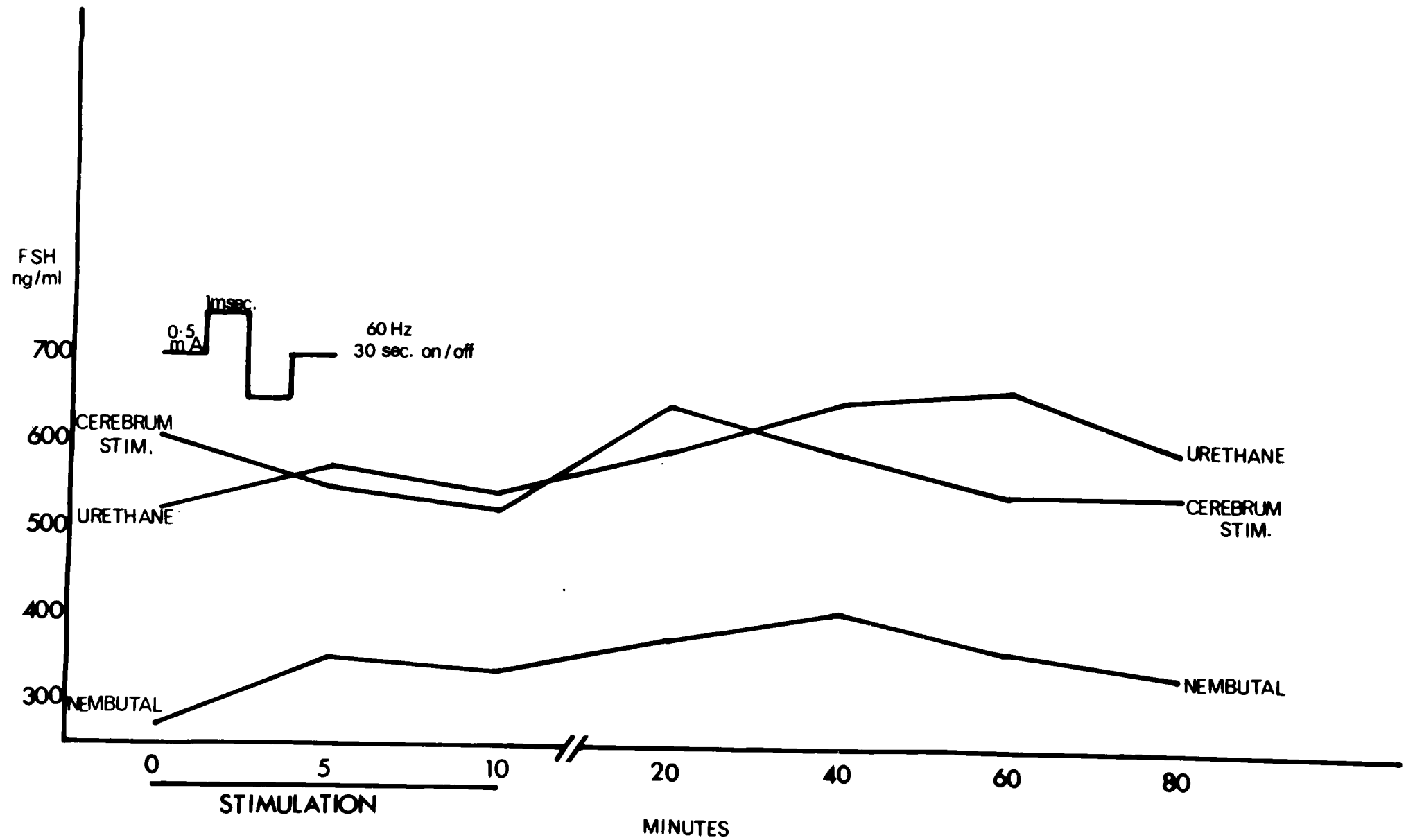


Figure A.6 Peripheral plasma FSH concentrations prior to, during and after; medial preoptic area a.c. stimulation under urethane or Nembutal anaesthesia, and cerebral a.c. stimulation (platinum electrodes).

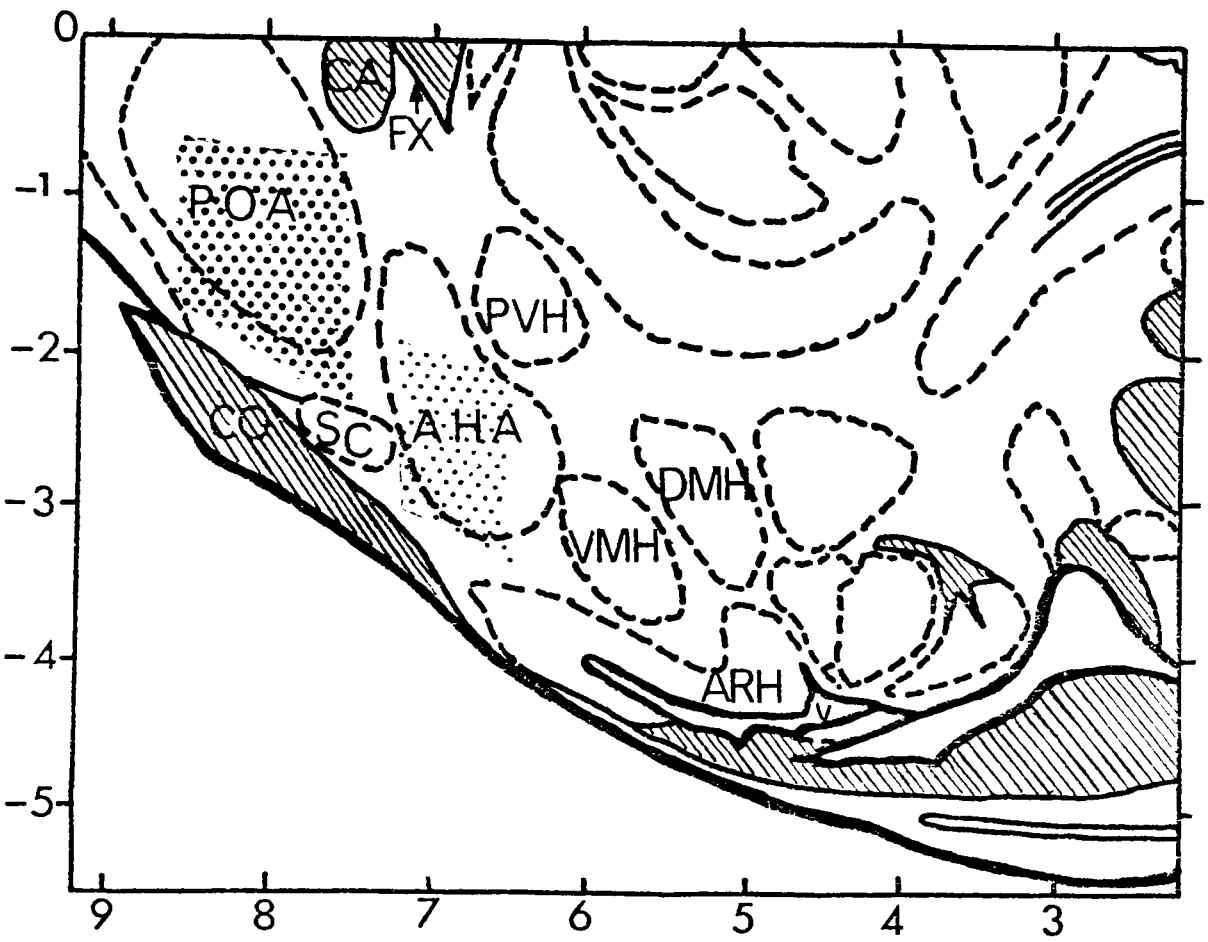


APPENDIX 2.4

Electrode Termination Sites

Histological verification of electrode termination sites in the medial preoptic or anterior hypothalamic areas was made according to Figure A.7.

APPENDIX 2.4(cont.)



medial preoptic area stimulation sites.

anterior hypothalamic area stimulation sites.

FIGURE A.7. Diagram of midline sagittal section of rat brain diencephalon (after de Groot, 1952) showing areas of Stimulation in experiments described in Chapters 2, and 4.

Key: AHA - Area anterior hypothalami; ARH - Nucleus arcuatus hypothalami; CA - Commissura anterior; CO - Chiasma opticum; DMH - Nucleus dorsomedialis hypothalami; FX - Fornix (corpus columna); POA - Area preoptica (medialis, lateralis); PVH - Nucleus paraventricularis hypothalami; SC - Nucleus supra-chiasmaticus; V - Ventriculus cerebri; VMH - Nucleus ventro-medialis hypothalami

APPENDIX 3.1

Composition of Minimum Essential Medium with Earle's Salts and 20 mM Hepes Buffer

Amino Acids	mg/litre
L-arginine	126.4
L-cystine	24.0
L-glutamine (added prior to assay)	292.0
L-histidine HCl	38.3
L-isoleucine	52.5
L-leucine	52.5
L-lysine	73.1
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.9
Vitamins	
D-calcium pantothenate	1.0
Choline chloride	1.0

APPENDIX 3.1 (cont.)

	mg/litre
folic acid	1.0
l-inisitol	2.0
nicotinamide	1.0
pyridoxal HCl	1.0
riboflavin	0.1
thiamine HCl	1.0

Inorganic salts and other components

$CaCl_2 . 2H_2O$	264.0
KCl	400.0
$MgSO_4 . 7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4 . H_2O$	140.0
n-2 Hydroxy ethylpiperazine ethanesulphonic acid pH 7.2 (HEPES)	4670.0
phenol red	17.0
glucose	1000.0

APPENDIX 3.2

Synthetic Peptides

Synthetic peptides related to the decapeptide LH-RF synthesised by classical methods by Drs. J. Gormley, H. Gregory, and A.M. Rowbotham of ICI Pharmaceuticals, Macclesfield.

LH-RF	1 - 10	□GLU-HIS-TRP-SER-TYR-GLY-LEU-ARG-PRO-GLY-NH_2
"	3 - 10	Z-TRP-SER-TYR-GLY-LEU-ARG-PRO-GLY-NH_2
"	7 - 10	H-LEU-ARG-PRO-GLY-NH_2
"	1 - 6	□GLU-HIS-TRP-SER-TYR-GLY-NH_2
"	1 - 8	□GLU-HIS-TRP-SER-TYR-GLY-LEU-ARG-NH_2
"	1 - 9	□GLU-HIS-TRP-SER-TYR-GLY-LEU-ARG-PRO-NH_2
"	3 - 9	Z-TRP-SER-TYR-GLY-LEU-ARG-PRO-NH_2
"	1-2+9-10	□GLU-HIS . . . . . PRO-GLY-NH_2
		□GLU-TYR-ARG-TRP-NH_2
		□GLU-GLN-ALA-NH_2

z = benzyloxycarbonyl-

APPENDIX 3.3

Worksheet for in-vitro Assay No. 13 - LH release

The calculation and notation is according to Bliss, (1952).

Treatment	Control	Dose LHRF ng/ml medium 7.5	30.0	Dose F_3 ng/ml medium 30	135
Response	1.95	5.30	14.40	6.10	10.80
ng LH/	1.77	10.00	9.30	4.35	7.40
ml medium	1.33	5.00	18.20	6.10	11.80
$x2x10^{-3}$	0.94	6.20	11.28	5.65	* 10.13
Mean ± S.E.M.	1.50 ± 0.22	6.62 ± 1.14	13.30 ± 1.94	5.55 ± 0.41	10.03 ± 0.94

t tests		t	
	Control vs LHRF 7.5 ng	4.37	$p < 0.01$
	Control vs F_3 30 ng	8.61	$p < 0.001$
	LRF 7.5 ng vs LHRF 30.0 ng	2.96	$p < 0.05$
	F_3 30ng vs F_3 135 ng	4.35	$p < 0.01$

For convenience in handling data, responses have been reduced by a factor of 500.

* Missing value.

Dose Response Curves

(A) Standard Doses 7.5 ng 30.0 ng
Logs 0.88 1.48.
S (x) = 9.44 $\bar{x}$ = 1.18
S (y) = 79.68 $\bar{y}$ = 9.96

(B) F_3 Let the doses be as for Standard.
S (x) = 9.44 $\bar{x}$ = 1.18
S (y) = 62.33 $\bar{y}$ = 7.79

APPENDIX 3.3 (cont.)

(x^2)	0.72	0.72
(y^2)	150.24	52.79
(xy)	7.98	5.35
b	11.23	7.53
a	-3.29	-1.09
B^2	89.69	40.31
$(y^2) - B^2$	60.55	12.48
S^2	10.09	2.50
F	8.89	16.12
λ	0.28	0.20
D.F.	6	5
p	<0.05	<0.05
Line	y = 11.23x - 3.29	y = 7.53x - 1.09

(C) Combined

b_c = 9.39

Bc^2 = 125.13

$(y^2)b$ = 4.69

S^2 = 6.63

F = 0.71 i.e. no significant difference between the slopes.

λc = 0.27

$Bc^2 - S^2t^2$ = 93.06

C^2 = 1.34

C = 1.16

APPENDIX 3.3 (cont.)

(D) Potency

M^1 = -0.23 S_M = 0.15

Add log of upper dose (1.48)

i.e. 1.25

Antilog 1.25 = 17.8

i.e. Potency of F_3 in terms of ng decapeptide (LH-RF)/ml medium = 17.8

(E) Confidence Limits

XL = -0.69 to +0.07

Add log of upper dose (1.48)

i.e. 0.79 to 1.55

Antilog 0.79 = 6.2

Antilog 1.55 = 35.5

(F) Conclusions

With respect to LH release, the potency (95 % confidence limits) of F_3 (LH-RF 1-9) in this assay in terms of ng decapeptide (LH-RF)/ml medium was 17.8 (6.2 to 35.5) when administered at a dosage of 135 ng F_3/ml medium. Potency ratio = 0.13.

APPENDIX 3.3 (cont.)

Worksheet for in vitro Assay No. 20 - LH release

Treatment	Control	Dose LHRF 7.5 ng/ml medium	30.0	Dose F_3 30 ng/ml medium	135
Response	1.47	6.10	11.00	4.00	* 6.27
ng LH/	1.57	7.00	8.40	2.22	5.20
ml medium	1.75	4.80	7.10	4.25	6.40
x 10^{-3}	1.82	5.10	10.40	3.50	5.20
Mean ± S.E.M.	1.58 ± 0.06	5.75 ± 0.50	9.23 ± 0.90	3.49 ± 0.45	5.77 ± 0.33

		t	
t tests	Control vs LH-RF 7.5 ng	8.34	$p < 0.001$
	Control vs F_3 30 ng	4.24	$p < 0.01$
	LHRF 7.5 ng vs LHRF 30.0 ng	3.38	$p < 0.02$
	F_3 30 ng vs F_3 135 ng	4.07	$p < 0.01$

For convenience in handling data, responses have been reduced by a factor of 1000.

* Missing value.

Dose Response Curves

(A) Standard Doses 7.5 ng 30.0 ng

Logs 0.88 1.48

S (x) = 9.44 $\bar{x}$ = 1.18

S (y) =59.90 $\bar{y}$ = 7.49

(B) F_3 Let the doses be as for Standard.

Sx = 9.44 $\bar{x}$ = 1.18

S(y) = 37.04 $\bar{y}$ = 4.63

APPENDIX 3.3 (cont.)

(x^2)	0.72	0.72
(y^2)	36.89	14.08
(xy)	4.17	2.73
b	5.79	3.79
a	0.66	0.16
B^2	24.15	10.35
$(y^2) - B^2$	12.74	3.73
S^2	2.12	0.75
F	11.39	13.80
λ	0.25	0.23
DF	6	5
p	<0.05	<0.05
Line	y = 5.79x + 0.66	y = 3.79x + 0.16

(C) Combined

b_c = 4.79

Bc^2 = 33.06

$(y^2)b$ = 1.44

S^2 = 1.50

F = 0.96 i.e. no significant difference between the slopes.

λc = 0.25

$Bc^2 - S^2t^2$ = 25.80

C^2 = 1.28

C = 1.13

APPENDIX 3.3 (cont.)

(D) Potency

M^1 = -0.60 S_M = 0.19

Add log of upper dose (1.48)

i.e. 0.88

Antilog 0.88 = 7.6

i.e. Potency of F_3 in terms of ng decapeptide (LH-RF)/ml medium = 7.6

(E) Confidence Limits

XL = -0.29 to -1.23

Add log of upper dose (1.48)

i.e. 1.19 to 0.26

Antilog 1.19 = 15.5

Antilog 0.26 = 1.8

(F) Conclusions

With respect to LH release, the potency (95 % confidence limits) of F_3 (LH-RF 1-9) in this assay in terms of ng decapeptide (LH-RF)/ml medium was 7.6 (1.8 to 15.5) when administered at a dosage of 135 ng F_3/ml medium. Potency ratio = 0.06.

APPENDIX 3.3 (cont.)

Worksheet for Assay No. 19 - FSH release

Treatment	Control	Dose LH-RF ng/ml medium 1.9	7.5	Dose F_3 ng/ml medium 30	135
Response	6.20	18.40	20.40	14.00	16.00
ng FSH/	7.00	14.40	* 24.20	12.20	22.20
ml medium	6.00	14.00	25.40	13.40	18.00
x 10^{-3}	6.40	18.00	34.00	I3.40	24.00
Mean ± S.E.M.	6.40 ± 0.20	16.20 ± 1.20	26.0 ± 2.90	13.25 ± 0.37	20.05 ± 1.80

t tests		t	
	Control vs LH-RF 1.9 ng	8.30	$p < 0.001$
	Control vs F_3 30.0 ng	14.60	$p < 0.001$
	LH-RF 1.9 ng vs LH-RF 7.5 ng	3.16	$p < 0.02$
	F_3 30 ng vs F_3 135 ng	3.77	$p < 0.01$

For convenience in handling data, responses have been reduced by a factor of 1000.

* Missing value.

Dose Response Curves

(A) Standard Doses 1.9 ng 7.5 ng
Logs 0.27 0.88
S (x) = 4.60 $\bar{x}$ = 0.58
S (y) = 168.80 $\bar{y}$ = 21.10

(B) F_3 Let the doses be as for Standard.
S (x) = 4.60 $\bar{x}$ = 0.58
S (y) = 133.20 $\bar{y}$ = 16.65

APPENDIX 3.3 (cont.)

(x^2)	0.74	0.74
(y^2)	307.2	135.02
(xy)	11.93	8.27
b	16.12	11.17
a	11.76	10.18
B^2	192.32	92.41
$(y^2) - B^2$	114.88	42.61
S^2	22.98	7.10
F	8.36	13.01
λ	0.30	0.24
DF	5	6
p	<0.05	<0.05
Line	y = 16.12x + 11.76	y = 11.17x + 10.18

(C) Combined

b_c = 13.64

B_c^2 = 275.70

$(y^2)b$ = 9.03

S^2 = 14.31

F = 0.63 i.e. no significant difference between the slopes.

λc = 0.27

$Bc^2 - S^2t^2$ = 206.45

C^2 = 1.34

C = 1.16

APPENDIX 3.3 (cont.)

(D) Potency

M^1 = -0.32 S_M = 0.16

Add log of upper dose (0.88)

i.e. 0.56

Antilog 0.56 = 3.6

i.e. Potency of F_3 in terms of ng decapeptide (LH-RF)/ml medium = 3.6

(E) Confidence Limits

XL = -0.85 to -0.03

Add log of upper dose (0.88)

i.e. 0.03 to 0.85

Antilog 0.03 = 1.1

Antilog 0.85 = 7.1

(F) Conclusions

With respect to FSH release, the potency (95 % confidence limits) of F_3 (LH-RF 1-9) in this assay in terms of ng decapeptide (LH-RF)/ml medium was 3.6 (1.1 to 7.1) when administered at a dosage of 135 ng F_3/ml medium. Potency ratio = 0.03.

APPENDIX 3.4

Radioimmunoassay Reagents

(1) 0.01 M Phosphate buffered saline, 0.1 % gelaxine 0.01 % merthiolate (gel - PBS)

NaCl	8.170 g/l
NaH_2PO_4	0.250 g/l
Na_2HPO_4	1.193 g/l
Merthiolate	0.100 g/l
Gelatine	1.000 g/l
pH	7.0

Stored at 0-4°C.

(2) 0.01 M Phosphate buffered saline, 0.01 % merthiolate 0.05 M EDTA, 0.043 % Normal Rabbit serum (antiserum buffer)

NaCl	8.170 g/l
NaH_2PO_4	0.250 g/l
Na_2HPO_4	1.193 g/l
Merthiolate	0.100 g/l
Na EDTA	18.61 g/l
Normal Rabbit serum	4.3 ml/l (1 : 233)
pH	7.0

APPENDIX 3.4 (cont.)

NOTES

Merthiolate is included as a preservative.

The method of separating bound from free hormone by the double antibody method requires the presence of some ethylene-diaminotetraacetic acid (EDTA) which inhibits the series of reactions that eventuate in the formation of complement, the presence of which may lead to spurious results.

Gelatine.

In the very low concentrations at which they are used, labelled and unlabelled hormone and the antibodies will tend to be absorbed to the reaction vessels. This absorption is reduced or prevented by including protein in the buffer solution.

Normal Rabbit Serum.

In the double antibody system, addition of further gamma globulin in the form of serum increases the size of the precipitate to the anti-gamma globulin. A good size precipitate is easier to handle. Serum is added from a normal animal of the species in which the first antibody was developed. It should be noted, however, that increasing the precipitate size increases the "blank" (N) value, and demands the presence of rather more anti-gamma globulin. In practice, it has been found that 0.043% normal rabbit serum in the antiserum buffer provides an adequate precipitate.

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