THE CONTROL OF LUTEINISING HORMONE SECRETION IN THE MARMOSET MONKEY, *CALLITHRIX JACCHUS*

by

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To my Parents

Statement as to the author's participation in the work submitted

The composition of the Thesis is that of the author. The author has acted as the principal investigator for these studies and has been personally involved in the design, organisation and conduct of the experiments. All radioimmunoassays have been carried out by the author. Contributions of technical assistance have been duly acknowledged.

Signed,

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PUBLICATIONS

The following publications have been produced in connection with this thesis:

Abstracts

J.K. Hodges (1977) Immunisation against luteinising hormone-releasing hormone (LH-RH) in the marmoset monkey, Callithrix jacchus. Proc. Sixth Cong. Int. Prim. Soc.

Full papers

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J.K. Hodges and J.P. Hearn. A positive feedback effect of oestradiol on LH release in the male marmoset monkey, Callithrix jacchus. J. Reprod. Fert. (In press).

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ABSTRACT OF THESIS

This is a study of the control of luteinising hormone (LH) secretion in the marmoset monkey, Callithrix jacchus. The way in which the hypothalamo-hypophysial-gonadal system operates to regulate LH secretion in primates is reviewed.

A double antibody heterologous radioimmunoassay for measuring LH in marmoset plasma was developed and validated.

Intra-muscular administration of synthetic luteinising hormone-releasing hormone (LH-RH) induces a marked increase in plasma LH concentrations in the marmoset, suggesting that the releasing hormone has an important physiological role in controlling LH secretion in this species. The effects of steroid hormones on pituitary response to exogenous LH-RH were examined. Pituitary responsiveness to LH-RH was enhanced in long term, but not in short term, gonadectomised animals. Whereas oestradiol-17β implants inhibited pituitary response to LH-RH in long term castrates, implants of progesterone augmented the response. A direct action of gonadal steroids on the pituitary gland is therefore suggested.

The way in which steroid hormones influence the hypothalamo-hypophysial system was further studied by examining their ability to depress or increase circulating LH concentrations. Gonadectomy resulted in an increase in plasma LH levels indicating that LH secretion is normally suppressed by the action of gonadal steroids. In the "open-loop" situation the elevated LH levels are the result of an episodic secretion of the hormone by the pituitary gland. Closure of this feedback loop with oestradiol-17β caused a chronic suppression of LH secretion, suggesting that this steroid is an important component

of the negative feedback mechanism regulating tonic LH secretion. The effects of progesterone, testosterone and dihydrotestosterone on LH secretion in gonadectomised marmosets were also tested. Whereas these hormones prevented the post-castration rise in LH concentrations, they were apparently ineffective in suppressing LH levels in long term gonadectomised animals. A decrease in the sensitivity of the hypothalamic-pituitary system to negative feedback as the interval from castration increases is suggested.

Positive feedback control of LH secretion was also examined. A single injection of oestradiol benzoate had a biphasic effect on LH secretion, with an initial negative feedback effect characteristically preceeding the positive response. Oestrogen induced LH release was observed in castrated and intact males as well as in castrated females, suggesting that the positive feedback response to oestrogen in the marmoset is not a sexually dimorphic characteristic. The ability of progesterone, testosterone, and dihydrotestosterone to induce, or modify oestrogen induced, positive feedback was also assessed in gonadectomised animals.

The effects of inhibition of LH-RH were also examined. Active immunisation against LH-RH induced a breakdown of the hypothalamo-hypophysial-gonadal system, resulting in inhibition of gonadal and pituitary function. LH-RH induced LH release was suppressed by the use of competitive antagonist analogues of LH-RH. The potential application of these procedures in fertility control is discussed.

CHAPTER ONE

REVIEW OF LITERATURE

1.1 Introduction

The concept of endocrine control of the gonads probably originates from an experiment performed by Hunter in 1787. Although the results were misinterpreted at the time, they did in fact show that hemiovariectomy in a sow led to compensatory hypertrophy and increased ovulation rate in the remaining ovary. Direct evidence for the involvement of the pituitary gland in gonadal function was not obtained until over 100 years later, when Fichera (1905) reported hypertrophy of the pituitary gland after castration. Following a series of classical experiments involving hypophysectomy and pituitary transplantation (Smith, 1926, 1927; Smith and Engle, 1927) the nature of the gonadotrophic stimulus was determined, and the existence of two distinct gonadotrophins - follicle stimulating hormone (FSH) and luteinising hormone (LH) - was finally confirmed by Fevold, Hisaw and Leonard in 1931. After many years of effort, purification and structural elucidation of LH from various species has now been achieved (see Sairam and Papkoff, 1974, for review).

Because the hypothalamus is the part of the brain nearest to the pituitary gland (Fig. 1.1), it was reasonable to envisage neural components in the control of pituitary hormone secretion. In 1936 Marshall reviewed the information on the effects of both physical and psychological factors on reproduction and postulated that the anterior pituitary might be under the control of substances manufactured in the brain and transported to the pituitary. Many suggested that there was a direct innervation, but the true relationship had been suggested earlier by Popa and Fielding (1930,

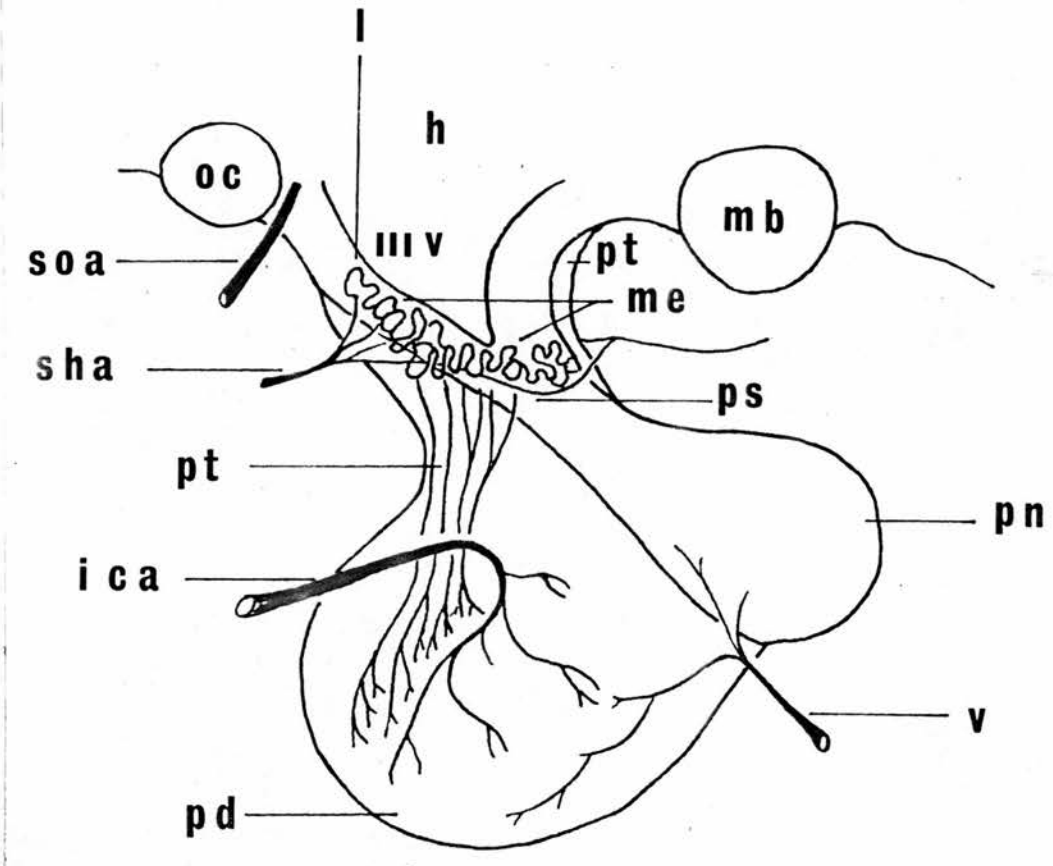


Figure 1

Diagram of a sagittal section through the hypothalamus and pituitary gland

oc	optic chiasm	soa	supra-optic artery
mb	mamillary body	l	sinusoidal loops
111v	third ventricle	sha	superior hypophysial artery
h	hypothalamus		
me	median eminence	pt	portal trunks
ps	pituitary stalk	ica	internal carotid artery
pt	pars tuberalis	v	venous sinus draining pituitary
pn	pars nervosa		
pd	pars distalis		

1933) who described a system of portal vessels connecting capillaries in the median eminence with the sinusoid spaces of the anterior pituitary. In his classic monograph Harris (1955) drew together all the evidence from electrical stimulation, ablation, stalk-sectioning and pituitary transplantation experiments and presented convincing evidence for the neurochemical control of the anterior pituitary via the portal vessels. Final confirmation of the neurohumoral control of the anterior pituitary came from Porter and Jones (1956), and four years later the presence of a luteinising hormone-releasing factor was first described by McCann, Taleisnik and Friedman (1960).

During the 1960's a great deal of effort was applied to the isolation and purification of luteinising hormone releasing factor. These attempts culminated in the simultaneous structural elucidation of porcine (Matsuo, Baba, Nair, Arimura and Schally, 1971) and ovine (Burgus, Butcher, Ling, Monahan, Rivier, Fellows, Blackwell, Vale and Guillemin, 1971) luteinising hormone releasing factor (now known as luteinising hormone releasing hormone, LH - RH) both of which were decapeptides with the amino acid sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH^2.

LH-RH has subsequently been synthesised (e.g. Matsuo et al., 1971; Sievertsson, Chang, Klaudy, Bogentoft, Currie, Folkers and Bowers, 1972), and Matsuo et al (1971) reported that their synthetic decapeptide showed the same physico-chemical and biological properties as their isolated natural porcine hormone.

The exact localisation of LH-RH synthesis remains unclear. The initial demonstration of a "hypophysiotrophic area" (HTA)

in the rat hypothalamus (Halasz, Pupp and Uhlarik, 1962) provided the basis for many early studies designed to delineate the sites of LH-RH production. Various techniques have been applied but unfortunately the data are rather confusing (see Hodges, 1975, for review). In recent years more sophisticated techniques (including autoradiography, immunofluorescence and immunocytochemistry) have been introduced, and although the results obtained using these methods confirm the importance of the HTA as the region of LH-RH synthesis, the actual distribution of LH-RH within the HTA seems to depend upon which method is employed (see Ramirez and Kordon, 1975; Dubois, 1976, for reviews).

It is now well established that gonadal steroids can regulate pituitary gonadotrophin secretion, and that depending upon the circumstances their influence can be either stimulatory or inhibitory. The concept of positive and negative feedback originated from observations early in the 1930's. In 1932 Moore and Price, and Hohlweg and Junkmann independently postulated a simple inverse relationship between circulating gonadal steroid concentrations and pituitary gonadotrophin secretion. This was deduced from their findings of gonadal atrophy following prolonged administration of oestrogen, and increased gonadotrophic activity after gonadectomy. Subsequently a great deal of information has accumulated regarding negative feedback and much of the earlier work has been reviewed (e.g. Hisaw, 1947; Van Rees, 1964). More recent studies, utilising radioimmunoassay methods, have demonstrated a marked increase in circulating LH levels following castration in either

sex of numerous species (see Labhsetwar, 1973, for review), providing conclusive evidence that pituitary LH secretion is normally suppressed by gonadal steroids.

The existence of a positive feedback effect of gonadal steroids was originally suggested when Hohlweg (1934) demonstrated that the administration of oestrogen resulted in the formation of corpora lutea in prepubertal rats. Following the observation by Everett (1948) that oestrogen could advance ovulation in the rat, Everett, Sawyer and Markee (1949) suggested that oestrogens acting on the central nervous system initiate the ovulatory release of LH through a positive feedback action. This has since been confirmed in a variety of species, including rats (Ferin, Tempone, Zimmering and Vande Wiele, 1969), sheep (Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969), rhesus monkeys (Ferin, Dyrenfurth, Cowchock, Warren and Vande Wiele, 1974) and women (Vande Wiele, Bogumil, Dyrenfurth, Ferin, Jewelewicz, Warren, Rizkallah and Mikhail, 1970). The existence of a dual feedback system regulating LH secretion is now well established. By acting at the hypothalamic and hypophysial levels gonadal steroids regulate LH secretion by means of negative (controlling tonic secretion) and positive (controlling cyclic release of LH) feedback mechanisms.

It is therefore possible to identify the three elements of the system which is concerned with regulating LH secretion; the central nervous system-hypothalamus complex may be regarded as a signal generator, the pituitary as a signal transmitter, and the gonadal steroid output as a signal modulator. Within this general context, the regulation of the hypothalamo-hypophysial-gonadal system in primates will be reviewed.

1.2 The hypothalamic-hypophysial system

Administration of natural or synthetic LH-RH will elevate plasma levels of LH, and in most cases FSH, due to direct stimulation of the anterior pituitary gland (Schally, Kastin and Arimura, 1971). This stimulatory effect of LH-RH involves stimulation of both release and de novo synthesis of LH and FSH by the pituitary gonadotrophs (Redding, Schally, Arimura and Matsuo, 1972).

The stimulatory action of LH-RH on LH secretion in primates has been described by numerous investigators (e.g. Jaffe and Keye, 1974; Wang, Lasley, Lein and Yen, 1976; Hoff, Lasley, Wang and Yen, 1977 - human: Krey, Butler, Weiss, Weick, Dierschke and Knobil, 1973; Ferin, Warren, Dyrenfurth, Vande Wiele and White, 1974 - rhesus monkey). Although a relative insensitivity to synthetic LH-RH has been reported in certain studies on the rhesus monkey (Ehara, Ryan and Yen, 1972; Arimura, Spies and Schally, 1973) it is unlikely that this represents species differences in endogenous LH-RH. Compelling evidence that the endogenous LH-RH in the rhesus monkey must closely resemble, at least immunologically, synthetic LH-RH has recently been provided by McCormack, Plant, Hess and Knobil (1977) who demonstrated a marked suppression of tonic LH secretion in ovariectomised animals following administration of antiserum to synthetic LH-RH.

1.2.a The control of tonic (pulsatile) LH secretion

The hypothalamus and the pituitary form a closely coupled functional unit which releases LH, often in the form of periodic pulses characterised by a high frequency and amplitude. Short term variations in LH secretion were first observed in gonadectomised

male and female rhesus monkeys, where the periodicity of episodic LH release was approximately one hour (Atkinson, Bhattacharya, Monroe, Dierschke and Knobil, 1970). A pulsatile pattern of LH release (with a frequency of 1-2 hours) has also been observed in hypogonadal (postmenopausal) and normal cycling women, and similar observations have been made in intact bulls (Katongole, Naftolin and Short, 1971), and castrated (Reeves, O'Donnell and Denorscia, 1972) and intact (Yuthasastrakosol, 1977) sheep. In the human female pulsatile LH secretion is seen during all phases of the menstrual cycle although there are significant variations in amplitude and frequency of the pulses throughout the cycle. During the luteal phase, frequency appears to be reduced as compared to the follicular phase, whereas amplitude is smaller during the late follicular phase than during the luteal phase or periovulatory period (Midgley and Jaffe, 1971; Yen, Vandenberg, Tsai and Siler, 1974). It is likely that these changes are caused by the steroid hormone environment during the different stages of the cycle.

The relationship between gonadal steroids and pituitary LH secretion has been investigated in part by measurements of the change in circulating LH concentrations following gonadectomy in male and female rhesus monkeys (Atkinson et al, 1970) and in premenopausal women (Yen and Tsai, 1971b; Monroe, Jaffe and Midgley, 1972). In both species LH concentrations increase markedly within 2-3 days after gonadectomy and continue to rise until a plateau is reached at approximately 10 times the initial concentration 3 weeks after the operation. It has been shown in the human that during the first week after ovariectomy, a significantly greater rise in LH is observed in those subjects

ovariectomised during the follicular phase than in those ovariectomised during the luteal phase of the menstrual cycle (Yen and Tsai, 1971b). Although indirect, these findings suggest that in the human changes in synthesis, storage and release of pituitary LH are influenced by circulating levels of oestrogen and/or progesterone.

The elevated LH levels in gonadectomised rhesus monkeys and humans appear to be maintained by an increase in the magnitude of pulsatile pituitary discharges of the hormone (Yen, Vandenberg, Rebar and Ehara, 1972; Santen and Bardin, 1973; Knobil, 1974). This accelerated release (and synthesis) of LH is probably, in large measure, determined by the hypophysiotrophic effect of an increased LH-RH secretion, since an elevated release of LH-RH in the absence of gonadal feedback has been found in both humans (Seyler and Reichlin, 1973) and rats (Ben-Jonathan, Mical, and Porter, 1973).

Although the control of the pulsatile discharge of LH from the adenohypophysis is not well understood it is reasonable to assume a causal pulsatile release of hypothalamic LH-RH. The finding of a pulsatile fluctuation of LH-RH in peripheral plasma of hypogonadal women (Seyler and Reichlin, 1974 a) and in the portal blood of rhesus monkeys (Carmel, Araki and Ferin, 1975), and the demonstration that antiserum to LH-RH abolishes pulsatile secretion of LH (McCormack and Knobil, 1975) add credence to this assumption. However, the precise neurons responsible for LH-RH synthesis and the controlling system for LH-RH secretion remain unclear.

Since it is established that catecholamines, present in abundance in the hypothalamus, serve as neurotransmitters for

critical steps in the regulation of hypothalamic LH-RH (Kamberi, Mical and Porter, 1971), the mechanism governing the pulsatile rhythm has been investigated through the use of adrenergic blocking agents. Pulsatile LH release is abolished by α (phentolamine) but not by β (propranolol) blocking agents in the ovariectomised rhesus monkey (Bhattacharya, Dierschke, Yamaji and Knobil, 1972), although this has not been confirmed in humans (Santen and Bardin 1973; Yen, Vandenberg, Tsai and Parker,1974). This discrepancy in findings may either be a consequence of dosage used (doses in the human studies were one tenth of those in the rhesus experiments), or possibly due to a partial blood-brain barrier to phentolamine. In any event, it has now been shown that catecholamines are important in the control of LH secretion in the human. Yen and his co-workers (Leblanc, Lachelin, Abu-Fadil and Yen, 1976; Lachelin, Leblanc and Yen, 1977) have clearly shown a marked inhibitory effect of dopamine and dopamine agonists (L-dopa and 2-bromo-α-ergocryptine) on LH secretion. The mechanism by which dopamine and dopamine agonists exert their inhibitory action is unknown. It may be due to a direct effect on hypothalamic LH-RH neurons as suggested by Fuxe, Hokfelt, Agnati, Lofstrom, Everitt, Johansson, Jonsson, Wuttke and Goldstein (1977), but a direct action on the pituitary responsiveness to LH-RH may also be operative.

While the exact nervous origin of the pulses in LH secretion remains uncertain, a few studies have been undertaken in primates in this direction. A type of experimental approach in rhesus monkeys involves the use of a stereotaxically introduced modified Halasz knife (Halasz and Gorski, 1967) to disconnect the

hypothalamus from other parts of the brain. The results, reported by Krey, Butler and Knobil (1975), indicate that centres controlling LH secretion may reside in an area of the hypothalamus closely related to the pituitary gland. The authors found that complete deafferentation of the medial basal hypothalamus in five ovariectomised rhesus monkeys did not inhibit the pulsatile release of LH, nor did it affect the mean plasma concentration of the hormone.

Although it is generally accepted that the hypothalamus and central nervous system are intimately involved in the control of pulsatile LH release, there is certain disagreement regarding the degree of autonomy of the pituitary gland in this respect. The demonstration of a pulsatile pattern of LH-RH activity in portal blood of rhesus monkeys (Carmel et al, 1975) suggests that secretion of LH by the pituitary may mirror the stimulation it receives from LH-RH. In addition, Osland, Gallo and Williams (1975) demonstrated that pulsatile LH release from superfused isolated rat pituitaries can be obtained only by pulsed delivery and not by constant infusion of LH-RH. In contrast however, Vande Wiele and Ferin (1974) reported that constant infusion of LH-RH to rhesus monkeys resulted in a pulsatile type of LH release, and that this effect was most pronounced in ovariectomised animals. It is possible, but unlikely, that these results are due to a pulsatile type of LH-RH metabolism. More realistically, the possibility exists that an endogenous pulsatile secretion of LH-RH is superimposed on the higher LH levels induced by the infusion. This hypothesis was in fact tested by sectioning the hypothalamic-pituitary stalk in an ovariectomised monkey, placing a silastic

barrier between the two cut portions, and measuring circulating LH levels during constant LH-RH infusion (Vande Wiele and Ferin, 1974). Surprisingly, the authors found that LH release remained pulsatile. Although this suggests that the anterior pituitary gland may be capable, by an unknown mechanism, of releasing LH in a pulsatile fashion, it is not known how long it is able to do so after separation from the brain. Since these results were derived from an experiment on a single animal, caution must be exercised in interpretation of these findings. Confirmation of an independent role of the pituitary gland in pulsatile release of LH will therefore have to await further in vivo and in vitro tests.

1.2.b The control of cyclic LH secretion

In females, superimposed upon the basic rhythm of LH secretion is a cyclic pattern of LH release during reproductive years associated with the menstrual cycle. The changes in LH secretion during the menstrual cycle have a periodicity of approximately one month, and appear to be consequent to cyclicity inherent in the secretory and gametogenic aspects of ovarian function (Vande Wiele et al, 1970; Yen et al, 1974b). The pattern of LH secretion during the menstrual cycle is basically similar in all species of higher primates, and has been described by numerous investigators (e.g. Swerdloff and Odell, 1968; Cargille, Ross and Yoshimi, 1969 - human: Monroe, Atkinson and Knobil, 1970; Hotchkiss, Atkinson and Knobil, 1971 - rhesus monkey: Goncharov, Aso, Cekan, Pachalia and Diczfalusy, 1976 - baboon: Wilks, 1977 - stump-tailed macaque). In the marmoset monkey menstruation does not occur and there is no externally obvious indication of oestrus.

In this species the "ovarian" cycle is considerably shorter (16-17 days) than the menstrual cycle of higher primates, although the cyclic pattern of LH secretion is broadly similar (Hearn and Lunn, 1975).

The patterns of LH secretion during the menstrual (human, rhesus, baboon, macaque) and ovarian (marmoset) cycles can be summarised; LH levels are relatively low during the early follicular phase of the cycle. LH secretion remains fairly low until just prior to ovulation when a peak in LH levels occurs (the pre-ovulatory surge). The midcycle LH peak is followed by a decline during the luteal phase to peripheral levels which are similar to, or slightly lower than those seen during the follicular phase.

The mechanisms responsible for the cyclic variation in LH secretion, in particular the midcycle LH surge, are complex and not yet fully understood. Steroid hormones play an important role in this respect, and will be dealt with later in this review. Of relevance to this section on the hypothalamic-pituitary axis is the variation in LH-RH output during the menstrual cycle. It is now generally accepted that the pre-ovulatory surge of LH is caused, in part, by an increased release of LH-RH from nerve terminals in the median eminence. In mature female rats it has been observed that hypothalamic LH-RH content is highest late in di-oestrus and declines at pro-oestrus (Chowers and McCann, 1965; Ramirez and Sawyer, 1965), suggesting that a release of LH-RH into the portal vessels may occur prior to and/or simultaneous with the ovulatory LH peak on the afternoon of pro-oestrus. However, initially, direct measurement of LH-RH in rat portal venous blood (Fink and Harris, 1976; Fink and Jamieson, 1976) on the day of pro-oestrus

did not reveal any significant increase in LH-RH concentrations. It has since been realised that as the anaesthetic used (urethane) can block ovulation, it will be likely also to block or truncate any surge of LH-RH. In a more recent study in which a different anaesthetic was used, an increase in LH-RH concentration in pituitary stalk blood has been shown to occur on the afternoon of pro-oestrus (Sarkar, Chiappa, Fink and Sherwood, 1976). In women, measurement of LH-RH by bioassay in peripheral blood revealed an increase on the day of the midcycle LH peak (Malacara, Seyler and Reichlin, 1972). This observation has since been confirmed using a radioimmunoassay (Arimura, Kastin and Schally, 1974).

It therefore seems likely that the preovulatory LH surge is associated with an increased LH-RH output. However, from quantitative measurements of LH-RH secretion during the pre-ovulatory period (Sarkar et al, 1976) it can be assumed that the increase in LH-RH secretion is alone, insufficient to cause the necessary rise in LH at mid cycle. Other factors are therefore involved and will be discussed later.

Prostaglandins are known to be involved in many reproductive processes (see Goldberg and Ramwell, 1975 and Roberts, Carlson and McCracken, 1976, for reviews), and there is now some evidence to suggest that LH secretion may be influenced by prostaglandins acting directly on the hypothalamic-hypophysial axis. Carlson, Wong and Perrin (1977) demonstrated in the rhesus monkey that prostaglandin E_2 or $F_2 \alpha$ will induce a small amount of LH release when administered in the luteal (but not follicular) phase, and that indomethacin (an inhibitor of prostaglandin synthesis) markedly reduces the amount of LH released in response to an

injection of oestradiol benzoate. Although the stimulation of LH release is not very convincing, and it may be argued that indomethacin produces other effects than inhibiting prostaglandin synthesis, these data suggest the possibility that prostaglandins may be involved in the induction of the pre-ovulatory LH surge. Where and how they act remains to be determined.

A summary of the hypothalamic-hypophysial function in the control of LH release is shown in Figure 1.2, which undoubtedly represents an oversimplification of a highly complex system.

1.3 The role of steroid hormones in the feedback modulation of LH secretion

While LH secretion is at all times directly related to LH-RH stimulation, the function of the hypothalamic-pituitary system is profoundly modified by the action of gonadal steroids. In theory any steroid which possesses some intrinsic oestrogenic, androgenic or gestagenic activity is capable of influencing pituitary LH secretion. Such steroids may be secreted directly by the gonads and/or adrenals, or they may arise from peripheral conversion of precursors, which themselves can be of either gonadal or adrenal origin. However, since many of the sex hormones secreted by the adrenal glands possess only weak intrinsic biological activity, and because the extent of precursor conversion is usually relatively small, it seems unlikely that the adrenals play a significant role in the feedback regulation of LH secretion. Thus under physiological conditions, those steroids most intimately involved in controlling LH secretion are probably entirely, or at least predominantly, derived from the ovaries or testes.

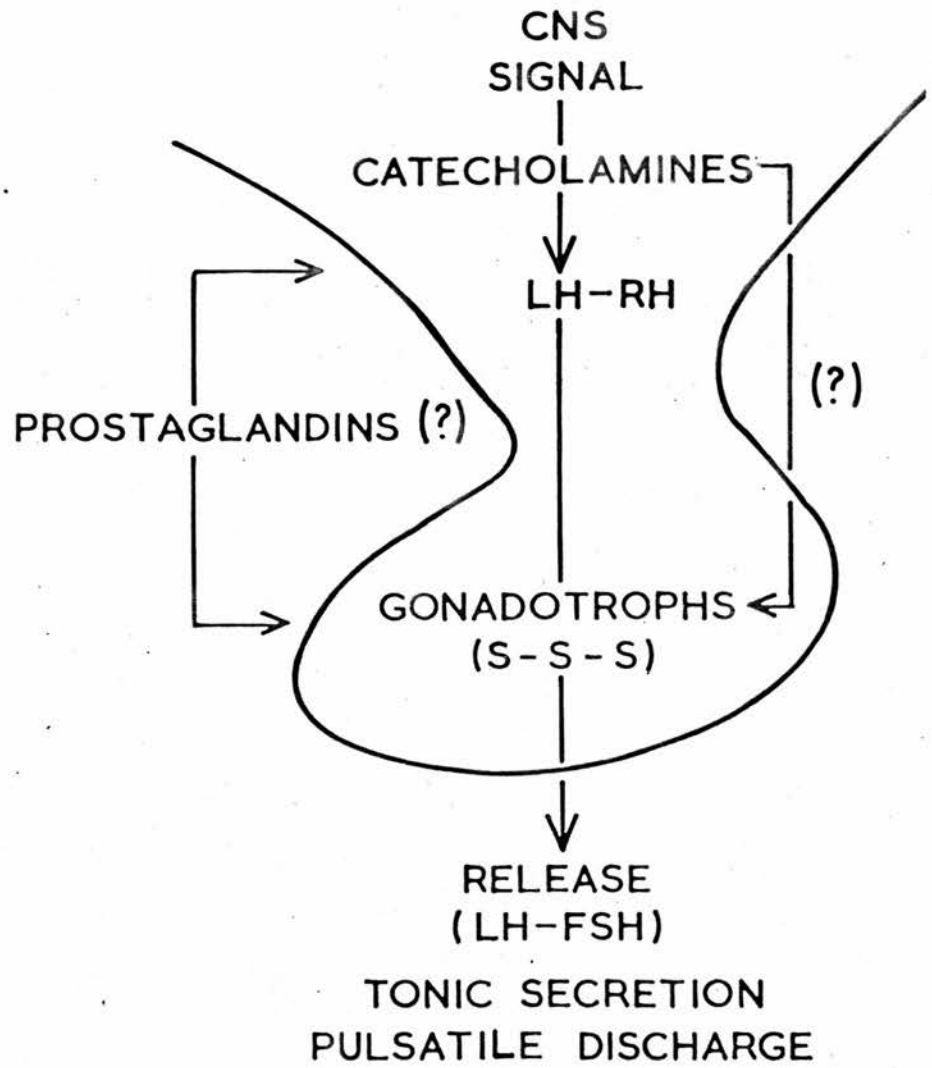


Figure 1.2.

Diagrammatic illustration of the possible sequence of events governing the pituitary discharge of gonadotrophins

(s-s-s = synthesis, storage and sensitivity of the gonadotrophs).
Modified from Yen et al, 1975.

1.3.a Steroid hormones which may control LH secretion and their site of origin

Most of the steroids in the biosynthetic pathway from pregnenelone to oestradiol-17β have been identified in the peripheral circulation of the human (Baird, 1974), rhesus monkey (Resko, 1971) and baboon (Goncharov et al, 1976). Confirmation of a direct ovarian secretion has been obtained for pregnenelone, progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, oestrone and oestradiol-17β in women (Baird, 1974), and for progesterone, testosterone and oestradiol-17β in the rhesus monkey (Hess and Resko, 1973). In all female primates studies so far peripheral concentrations of oestradiol-17β, progesterone and androstenedione show the most marked changes during the cycle, and hence are probably the most important with respect to feedback control of LH secretion.

In females of reproductive age circulating oestradiol-17β is almost exclusively derived from direct ovarian secretion. In the absence of bilateral ovulation, the majority of oestradiol-17β is produced by the ovary containing the pre-ovulatory follicle or corpus luteum (Baird and Fraser, 1974; Baird, Baker, McNatty and Neal, 1975 - human; Hess and Resko, 1973 - rhesus monkey). The remaining oestradiol-17β is derived from extraglandular conversion of oestrone (Baird, Horton, Longcope and Tait, 1969 - human; Resko, 1971 - rhesus monkey).

During the early follicular phase of the cycle circulating progesterone arises from at least three sources - direct ovarian and adrenal secretion, and extraglandular conversion of pregnenelone (Baird, 1974 - human; Bosu, Johansson and Gemzell, 1973 - rhesus

monkey). During the luteal phase of the cycle however, adrenal secretion and extraglandular conversion contribute very little to the total secretion rate, and virtually all of the progesterone is now secreted by the ovary containing the corpus luteum.

Of the androgens secreted by the female, androstenedione shows the most marked variation throughout the menstrual cycle and a midcycle rise has been detected in the human (Vermeulen and Verdonck, 1976) and the baboon (Goncharov et al, 1976). During most of the cycle, the adrenal gland is the major source of androstenedione, although in women the relative adrenal contribution decreases at midcycle (Baird, 1974). A recent report by Martensz, Baird, Scaramuzzi, and Van Look (1976) suggests that androstenedione may play an important role in controlling the pre-ovulatory LH release. These workers showed that immunisation against androstenedione inhibits the oestrogen induced LH surge in anoestrous sheep.

Comparisons of the basal concentrations of steroid hormones in the spermatic vein with those observed in the peripheral circulation indicate a direct testicular secretion of a wide range of steroids in man (Vihko and Ruokonen, 1974; Pazzagli, Borrelli, Forti and Serio, 1974). Apart from the testicular production of unconjugated steroids, the human testis has recently been shown to secrete considerable quantities of sulphate-conjugated steroids (Vihko and Hammond, 1976), although the physiological significance of these secretions largely remains undetermined.

The most important androgen secreted by the testis, both qualitatively and quantitatively, is testosterone. Small amounts

of testosterone may also be derived from direct adrenal secretion or from peripheral conversion of precursors. In the human, dihydrotestosterone is derived mainly from the testis (Pazzagli et al, 1974; Vihko and Hammond, 1976) although small amounts are also produced from peripheral conversion of testosterone and androstenedione (Mahodeau, Bardin and Lipsett, 1971; Pazzagli et al, 1974). Dihydrotestosterone is a potent androgen and together with testosterone probably represents the major component of the feedback mechanism controlling LH secretion in the male.

Although it is well established that oestrogens are secreted by the male (Baird, Galbraith, Fraser and Newsam, 1973; De Jong, 1974 for review), little is known about the role of endogenous oestrogens in controlling LH secretion.

No quantitative studies on in vivo testicular steroid production in sub-human primates have been reported, although in the rhesus monkey (Hoschoian and Brownie, 1967) and the marmoset (Preslock and Steinberger, 1977), production of testosterone, androstenedione, dehydroepiandrosterone and 17α-hydroxy-progesterone from simple precursors has been demonstrated in vitro.

To summarise, in the female, oestradiol-17β and progesterone are probably the most important steroids with regard to the feedback regulation of LH secretion. The role of other ovarian and adrenal steroids is not yet known although the possibility exists that some of these hormones, in particular androstenedione, may have a permissive action. In the male, testosterone and dihydrotestosterone appear to be the androgens most intimately involved in controlling LH secretion.

1.3.b Feedback mechanisms

Steroid hormones are known to exert both negative and positive feedback actions on LH secretion, and for many years numerous attempts were made to sort out these effects. However, owing to methodological problems, the information was necessarily limited (see Vande Wiele et al 1970, for review). With the advent of radioimmunoassays, a renewed effort was made and a clearer picture of the quantitative and qualitative aspects of these feedback mechanisms emerged.

In primates (Kelch, Kaplan and Grumbach, 1973; Dierschke, Karsch, Weick, Weiss, Hotchkiss and Knobil, 1974), as well as in rats (McCann, Ojeda and Negro-Vilar, 1974), negative feedback mechanisms are present from an early age, although it is generally believed that the sensitivity of the pituitary-hypothalamus complex to negative feedback action of steroids is lower after the onset of puberty (see Schönberg, 1975; Grumbach, Roth, Kaplan and Kelch, 1974; McCann, et al, 1974, for reviews). The finding that in pre-pubertally castrated rhesus monkeys (Dierschke et al, 1974) and children with gonadal dysgenesis (Conte, Grumbach and Kaplan, 1975), plasma LH levels rise at the age of expected puberty indicates that the pubertal change in hypothalamic feedback sensitivity occurs independently of the presence of functional gonads.

The dynamics of the negative feedback control of LH secretion has been examined in depth in the rhesus monkey. Yamaji, Dierschke, Bhattacharya and Knobil (1972) clearly demonstrated that brief pulses of oestrogen, achieved by single injections of oestradiol-17β or physiologic step increases of the steroid

effected and maintained by constant infusion, arrest within minutes the pulsatile discharges of LH observed in ovariectomised rhesus monkeys, with a resultant decline in plasma LH concentrations. The duration of this inhibition persists for several hours beyond the time when oestrogen ceases to be detectable in the peripheral plasma. It is of interest in this regard that in the ovariectomised rat, the hypothalamus and pituitary bind tritiated oestradiol-17β for approximately six hours after a single injection of the labelled steroid (McGuire and Lisk, 1968).

In 1973 a more detailed analysis of the negative feedback control of LH secretion in the rhesus monkey was performed by Karsch, Weick, Hotchkiss, Dierschke and Knobil. These workers showed that maintenance of circulating oestradiol concentrations within the range observed before ovariectomy (50-80 pg/ml)was usually ineffective in preventing the rise in circulating LH following ovariectomy, but that slightly higher levels (100-150 pg/ml) were effective. This observation together with the fact that step increments or decrements of as little as 20-30 pg/ml from threshold oestradiol concentrations elicited large (but reversible) changes in circulating LH levels, suggests that the negative feedback control system governing LH secretion is remarkably sensitive to seemingly small changes in plasma oestradiol concentrations.

A similar inhibitory effect of oestradiol on LH secretion has been demonstrated in the human female. Vande Wiele et al (1970) continuously infused oestradiol into menopausal women at a rate that approaches the physiological rate of secretion in the early follicular phase (100-150 ug/24 hours) and observed a

significant depression of LH levels. Oral administration of oestradiol-17β is also followed by a prompt decline in circulating LH levels (Yen, Martin, Burnier, Czekala, Greaney and Callantine, 1975).

During the follicular phase in women, most of the circulating oestradiol-17β is derived from the developing follicle (Baird and Fraser, 1974), and the negative feedback action of oestradiol during this phase is responsible for suppressing gonadotrophin secretion and therefore inhibiting further growth of other developing follicles. During the luteal phase oestradiol-17β which is now mainly secreted by the corpus luteum (Mikhail, 1970; Baird and Fraser, 1975) acts together with progesterone to suppress the release of gonadotrophins and inhibit follicular development. It is not until the corpus luteum regresses that the concentration of gonadotrophins is sufficiently high to initiate the development of a new 'wave' of Graafian follicles. The secretion of oestradiol-17β by the corpus luteum may therefore be one of the main factors determining the length of the follicular phase in women (Baird et al, 1975).

In contrast to the efficacy of oestrogen, progesterone by itself appears to be relatively inert with respect to a negative feedback action on LH secretion. In the ovariectomised rhesus monkey administration of progesterone in doses which achieve luteal phase concentrations does not result in a decrease in circulating LH levels (Karsch et al, 1973a). Furthermore Yamaji et al (1972) demonstrated that supraphysiological levels of progesterone, even when maintained for long periods, are also inactive in influencing LH secretion in ovariectomised rhesus monkeys. Similarly, the

injection of 10-100 mg of progesterone into postmenopausal women produced no decrease in circulating LH levels (Franchimont and Legros, 1970; Nillius and Wide, 1971). Recently Karsch, Foster, Legan and Hauger (1976) have questioned the significance of results obtained in long term ovariectomised animals, particularly with respect to the negative feedback action of progesterone in the ewe. These workers have demonstrated that although progesterone does not markedly depress LH levels in the long term ovariectomised ewe, the same dose of steroid when given as replacement treatment will prevent the post castration rise in LH secretion. A further report (Foster and Karsch, 1976) describes a similar inhibitory action of progesterone on tonic LH secretion in intact and ovariectomised immature ewes. Thus in contrast to the observations in primates, progesterone by itself appears to have a negative feedback effect on tonic LH secretion in female sheep. A species difference in the feedback role of progesterone may exist, although preliminary observations (this thesis, p.165) suggest that progesterone may inhibit LH secretion in the marmoset. Further investigation into the negative feedback action of progesterone in primates is obviously required, and this subject will be discussed further in chapter 5 of this thesis.

Although in most cases progesterone alone seems to have no significant inhibitory effect on LH secretion, physiological amounts of progesterone can synergise with sub-threshold (i.e. ineffective) levels of oestradiol-17β in the inhibition of tonic LH secretion in ovariectomised rhesus monkeys (Karsch et al, 1973a). A similar synergistic action of synthetic oestrogens and gestagens in suppressing pituitary LH secretion can be demonstrated in agonadal

women (Wallach, Root and Garcia, 1970).

Little is known concerning the role of androgens in the regulation of LH secretion in the female, although Martensz et al (1976) demonstrated that immunisation against androstenedione in the anoestrous ewe increased the frequency of pulsatile LH discharges and elevated mean LH concentrations compared to non-immunised controls.

In the male testosterone and dihydrotestosterone are considered to be the main androgens controlling LH secretion, but few attempts have been made to quantify their effects. Testosterone administration to normal men, either by intramuscular injection (Lee, Jaffe, Midgley, Kohen and Niswender, 1972) or by infusion at physiological secretion rates (Sherins and Loriaux, 1973; Stewart-Bentley, Odell and Horton, 1974), produces a dose related fall in LH levels. In contrast, physiological doses of testosterone are ineffective in depressing plasma LH concentrations in long term castrated male rhesus monkeys, and are equally ineffective in preventing the post castration rise in LH in these animals (Resko, Quadri and Spies, 1976). It is unlikely that this discrepancy is entirely due to species difference but may simply be a reflection of differing experimental procedures in terms of method, rate and duration of testosterone administration. More recently, the same group of workers (Resko, Quadri and Spies, 1977) has confirmed that within the confines of the experimental protocol, testosterone alone is unable to suppress LH in either long-term or short-term gonadectomised male rhesus monkeys. They have however provided evidence for a synergistic role for testicular oestradiol-17β

with testosterone by showing that a combination of subthreshold amounts of oestradiol and physiological levels of testosterone will maintain precastration levels of LH in short-term gonadectomised males.

It is possible however, that it is not testosterone itself that operates the feedback mechanism but another steroid to which testosterone may be converted. Administration of dihydrotestosterone (a 5α -reduced metabolite of testosterone) in man suppresses LH secretion, although not as effectively as testosterone (Stewart-Bentley et al, 1974). Oestradiol-17β can also inhibit LH secretion in men (Stewart-Bentley et al, 1974; Wang, Lasley and Yen, 1975) although the physiological role of oestrogens in the negative feedback control of LH in the male is not fully understood.

It is well established that the pre-ovulatory rise in circulating oestradiol-17β represents the critical stimulus for the initiation of the midcycle LH surge in women (Vande Wiele et al, 1970) and rhesus monkeys (Yamaji et al, 1971; Ferin et al, 1974a), as well as in rats (Ferin et al, 1969) and sheep (Goding et al, 1969). Maturation of this type of response to oestrogen (positive feedback) seems to occur at a much later age in primates than in the other species. In rats (Ying, Fang and Greep, 1971) and sheep (Land, Thimonier and Pelletier, 1970) oestrogen induced LH surges can be evoked in immature animals, whereas in the rhesus monkey oestrogen induced LH surges are not demonstrable until 4-8 months after the menarche, and spontaneous cyclic LH surges (resulting in ovulation) do not occur until even later (Dierschke et al, 1974a). Similar information suggests a late maturation of the positive feedback

mechanism in girls (Reiter,Kulin and Hamwood, 1974; Presl, Horejsi, Stroufova and Herzmann, 1976). Thus in primates maturation of the positive feedback mechanism appears to be a late pubertal event. Furthermore, the maturation process appears to be independent of gonadal steroid secretion, since it cannot be advanced in pre-pubertal female rhesus monkeys by chronic treatment with oestrogen or progesterone (Dierschke et al, 1974b).

The late maturation of the positive feedback mechanism (and hence the ability to ovulate) results in a period of post pubertal infertility, which may well turn out to be a uniquely primate phenomenon. It certainly occurs in chimpanzees in the wild, where 1-2½ years may elapse between menarche and the first conception (McGinnis, 1973); during this time the animals will show regular periods of oestrus with frequent copulation (Tutin, 1975). It could have been particularly valuable in primitive human communities since it would have allowed sufficient time for sexual exploration and the establishment of a lasting pair bond before the first conception (Short, 1976). Whether post-pubertal infertility is still of advantage in developed countries today where social and moral attitudes have changed dramatically is more doubtful.

Since pre- or neonatal androgen exposure of females of both rhesus monkey (Goy and Resko, 1972; Treolar, Wolf and Meyer, 1972) and human (Wilkins, 1965) species appears not necessarily to affect normal ovarian cyclicity (i.e. response to positive feedback) in later life, it has been suggested that unlike rodents (Gorski, 1971; Caligaris, Astrada and Taleisnik, 1971) or sheep (Short, 1974; Karsch and Foster, 1975; Clarke, Scaramuzzi and Short, 1976), the ability of the hypothalamic-pituitary unit to

discharge LH in response to oestrogen may not be a sexually dimorphic characteristic in primates. However, the data on this point are inconclusive. Initial attempts (Yamaji et al, 1971) to obtain a female-like LH surge in castrated male rhesus monkeys in response to a single injection of oestrogen were unsuccessful. It was only later (Karsch et al, 1973d), when LH levels in castrated animals were chronically suppressed with oestrogen before the oestradiol benzoate injection, that a positive feedback response was obtained. In such a situation however, the fact that the animals have been so far removed from their normal endocrine state makes interpretation of the results difficult. Furthermore, oestrogen induced LH release in intact male rhesus monkeys has not yet been demonstrated. It has been claimed that oestrogen may be capable of inducing LH release in normal (Kulin and Reiter, 1976) and homosexual men (Dörner, Rohde, Stahl, Krell and Masius, 1975), although the LH rises that were described are comparable in neither magnitude nor duration to those seen in normal women. A more detailed study by Van Look (1976) provided no evidence for the presence of a positive feedback effect of oestrogen in either normal or hypogonadal men.

Thus the data from men and intact rhesus monkeys provide little support for the existence of a positive feedback mechanism in male primates. In contrast however, recent observations in the marmoset monkey (Hodges and Hearn, 1978; this thesis, chapter 6) indicate oestrogen induced LH release in castrated and intact males. Whether such a response is unique to marmosets or is in fact present in other primate species has yet to be determined, and this will be discussed fully in chapter 7 of this thesis.

The hormonal requirements for the initiation of the pre-ovulatory LH surge are complex, and it is only fairly recently that any detailed information concerning the dynamics of positive feed-back in primates has been obtained. In the rhesus monkey, the strength duration characteristics of the increments in circulating oestrogen required to elicit an LH surge have been investigated by Karsch, Weick, Butler, Dierschke, Krey, Weiss, Hotchkiss, Yamaji and Knobil (1973). During the early follicular phase of the menstrual cycle plasma oestradiol concentrations below 100 pg/ml were ineffective in inducing LH surges, even when applied for as long as 120 hours. Similarly oestrogen concentrations of 100-200 pg/ml were incapable of inducing a positive LH discharge when applied for less than 42 hours. However, threshold doses of oestradiol (200-400 pg/ml) when maintained for 36 hours will consistently induce an LH surge.

Although the minimum requirements for an oestrogen stimulus to be effective in the human female are similar, a longer exposure time to the oestrogen stimulus (3-4 days) is required to consistently evoke an LH discharge (e.g. Yen and Tsai, 1972; Yen et al, 1974b; Van Look, 1976). In addition, Yen and his co-workers (Tsai and Yen, 1971a; Yen, Tsai, Vandenberg and Rebar, 1972) have demonstrated that although oestrogen administration during the mid follicular phase of the cycle elicits an acute release of LH, this does not occur when oestrogen is given in the early follicular phase. This would suggest that the hypothalamic-pituitary system becomes increasingly responsive to the positive feedback of oestrogen as the concentration of circulating (endogenous) oestrogen increases during follicular development. However this explanation does not

seem to apply to the rhesus monkey in which oestrogen induced LH surges can be regularly evoked either in the early follicular phase or in ovariectomised animals (Karsch et al, 1973c; Clifton, Steiner, Resko and Spies, 1975). Further investigation in the rhesus monkey may reveal that the threshold dose of oestrogen required to elicit an LH surge differs slightly at different stages of follicular development.

The stimulatory effect of oestradiol can be modified by several factors, the most important of which (with respect to the control of LH secretion) is probably progesterone. Administration of progesterone will block the positive feedback action of oestradiol in women (Netter, Gorius, Thomas, Cohen and Joubinaux, 1973) and intact rhesus monkeys (Dierschke et al, 1973; Clifton et al, 1975). The spontaneous ovulatory surge can also be prevented by continuous treatment of regularly cycling women with low doses of synthetic gestagens (e.g. Weiner, Johansson and Wide, 1976). This inhibitory effect of progesterone on oestrogen induced LH release may account for the failure to demonstrate oestrogen induced positive feedback during the luteal phase of the rhesus menstrual cycle (Dierschke et al, 1973). In contrast however, in ovariectomised rhesus monkeys, simultaneous administration of progesterone and oestradiol significantly advanced (rather than inhibited) the onset of LH release (Clifton et al, 1975). The physiological significance of this observation is difficult to assess, although the authors suggest it may indicate that the ovary is necessary, either directly or indirectly, for the blocking effect of progesterone.

Although progesterone by itself is not capable of inducing positive feedback in primates, administration of this steroid will

stimulate an LH surge providing oestrogen is also present. Thus progesterone can trigger an acute surge of LH after oestrogen priming in agonadal or post menopausal women (Odell and Swerdloff, 1968; Leyendecker, Wardlaw and Nocke, 1972) and castrated men (Stearns, Winter and Faiman, 1973), and a similar effect has also been demonstrated during the late follicular phase in normally cycling women (Yen, Lasley, Wang, Leblanc and Siler, 1975). Administration of 10 mg progesterone during the late follicular phase was found to induce a relatively brief (12 hr) surge of LH. This facilitatory action of progesterone was not demonstrable in the low oestrogen phase of the cycle, but in the high oestrogen phase appears to be operative in relatively low serum concentrations (1-2 ng/ml) with a short latency of approximately 4-6 hours. Since in the human plasma progesterone concentrations have been shown to be significantly elevated at the time of the midcycle LH surge (Johansson and Wide, 1969; Abraham, Odell, Swerdloff and Hopper, 1972) the possibility that progesterone acts synergistically with oestradiol must be considered.

From the available evidence it may be concluded that in the intact female primate oestradiol-17β provides the principal stimulus for inducing the positive feedback response which results in the preovulatory LH surge. The exact role of progesterone, or indeed any other gonadal steroid, in relation to the positive feedback action of oestradiol at midcycle remains to be determined.

1.4 The site of action of steroid hormones in the feedback control of LH secretion

During the past few years a major issue has been whether

the feedback modulating function of steroid hormones acts on the hypothalamus by changing the release of LH-RH, or on the pituitary by modifying its sensitivity to LH-RH, or a combination of both of these effects.

Since the existence of specific receptor sites for gonadal steroids in the adenohypophysis is well established (see Stumpf, Sar and Keefer, 1975, for review) a direct feedback action on the pituitary gland seemed likely. In vitro, oestradiol, or a combination of oestradiol and progesterone have been shown to suppress LH release in response to LH-RH (Schally, Redding and Arimura, 1973). Early in vivo attempts to test pituitary sensitivity to synthetic LH-RH during different phases of the menstrual cycle (e.g. Yen, Tsai, Naftolin, Vandenberg and Ajabor, 1972; Nillius and Wide, 1972 - human: Krey et al, 1973 - rhesus monkey) revealed that a "window" of maximal sensitivity to LH-RH occurs at midcycle. It seems likely that this apparent increase in pituitary sensitivity to LH-RH stimulation is a result of the feedback action of increased levels of circulating oestrogen. The finding that pituitary responsiveness to LH-RH during the mid luteal phase is at least as great as that seen in the late follicular phase (Yen et al, 1972a; Krey et al, 1973) suggests that the low circulating concentrations of LH typically found during the luteal phase of the cycle are not due to a pituitary insensitivity to LH-RH, but are perhaps the result of a decrease in the secretion of hypothalamic LH-RH which in turn may be occasioned by an inhibitory action of oestrogen and progesterone.

Initial attempts to demonstrate oestrogen feedback directly on the pituitary yielded puzzling results. Oestrogen administration in the human was found to reduce rather than increase pituitary responsiveness to LH-RH (Thompson, Arfania and Taymor, 1973; Keye and Jaffe, 1974). However these results are somewhat difficult to interpret because time and dose related factors were not considered. The effect of acute and chronic oestradiol administration on pituitary response to LH-RH has been further studied. Rapid increments in circulating oestradiol to levels of 700-900 pg/ml achieved by constant infusion of oestradiol-17β into hypogonadal women induced a marked diminution in pituitary response to LH-RH (Yen, Vandenberg and Siler, 1974). Short term exposure to oestradiol-17β (approximately 100 pg/ml) also resulted in a marked reduction in LH release in response to intrapituitary LH-RH infusion in the rhesus monkey (Spies and Norman, 1975). On the other hand, a more prolonged exposure of the pituitary to low levels of oestradiol appears to initially enhance (after 1 week) pituitary sensitivity to LH-RH, followed by a progressive inhibition (Yen et al, 1974c). These findings lend support to the concept that changes in pituitary sensitivity to LH-RH (during the menstrual cycle) are at least partially determined by temporally significant changes in oestradiol levels. Since exogenous oestrogen can reduce or enhance pituitary sensitivity to LH-RH its role in modulating LH secretion is obviously complex. Careful attention must be paid to the strength and duration characteristics of the oestrogen stimulus before the true situation in the menstrual cycle is revealed.

Evidence that oestrogen is responsible for changes in pituitary sensitivity has also been provided by the demonstration that the usually augmented response of the pituitary to LH-RH seen during high oestrogen phases of the menstrual cycle is completely eliminated by the administration of clomiphene (Wang and Yen, 1975). Clomiphene also markedly reduces pituitary release of LH in response to LH-RH in normal men (Wang et al, 1975). Since it has been shown that the oestradiol binding capacity is several times greater for the pituitary than for the hypothalamus (e.g. Korach and Muldoon, 1974), and also the competition of clomiphene for oestradiol receptors is greater at the level of the pituitary (Maurer and Woolley, 1971), the principal effect of the reduced LH-RH responsiveness is likely to be due to the elimination of the oestrogen effect by clomiphene on the pituitary, and the hypothalamic contribution to such an event is probably small. These interpretations must be viewed with caution, and considerations of dose and duration of clomiphene treatment should be given. In this regard, an augmented pituitary response to LH-RH has been found by Gonzalez-Barcena, Kastin, Schalch, Lee, Lander, Siller, Torres-Zamora, Rivas and Schally (1974) after the administration of a larger dose and longer duration of clomiphene to male subjects. At any rate it appears that steroid hormones exert part of their feedback action through a direct effect on the pituitary.

In addition, there is now convincing evidence to show that steroids, or more precisely oestradiol-17β , also modulate LH secretion by acting on the hypothalamus. As in rodents (Szentagothai, Flerko, Mess and Halasz, 1972, for review), the

medial basal hypothalamus (MBH) appears to be an important site of negative feedback action in the rhesus monkey. In 1972 Bhattacharya, Dierschke, Yamaji and Knobil demonstrated that α adrenergic blocking agents could simulate the negative feedback action of oestradiol-17β . More precise localisation of neural sites involved in the negative feedback effects of gonadal steroids was obtained by Ferin, Carmel, Zimmerman, Waren, Perez and Vande Wiele (1974). These workers were able to depress circulating LH levels by the injection of oestradiol-17β into various regions of the MBH (including the supra-chiasmatic, infundibular, ventromedial and the mammillary complex nuclei), but in general no effect was observed when oestradiol was applied to other hypothalamic or to extrahypothalamic sites. These findings have recently been confirmed by Spies, Norman, Quadri and Clifton (1977) who demonstrated that oestrogen inhibits LH release in response to MBH-electrical stimulation.

With regard to positive feedback however, the work of Krey, Butler and Knobil (1975) suggests that fundamental differences may exist between rodents and primates. In contrast to the rat, complete deafferentation of the MBH did not prevent spontaneous ovulation in the rhesus monkey, suggesting that the ovulatory discharge of LH in the rhesus monkey might not require a signal generated by the pre-optic area of the brain. However, Spies et al (1977) were unable to demonstrate a positive effect of oestrogen on the LH response to MBH-electrical stimulation in the rhesus monkey, despite a wide range of physiological oestradiol levels and intervals of exposure. It may be possible that shorter oestrogen treatment (less than 24 hours) would facilitate electrically

induced LH release, although this rationale is inconsistent with the oestrogen regimen required to induce an LH surge (Karsch et al, 1973c). Furthermore, these workers (Spies et al, 1977) did find that oestrogen treatment induced a small but unambiguous facilitation of LH release in response to electrical stimulation of the rostral hypothalamus (which includes the preoptic-suprachiasmatic region). Since Knobil's work (Krey et al, 1975) suggests that the MBH, and not the pre optic area is responsible for the expression of oestrogen induced LH release, the precise area controlling positive feedback in primates remains in doubt. The situation is obviously complex and requires further detailed investigation.

The above studies provide conclusive evidence that gonadal steroids exert feedback effects at the hypothalamic level. That the positive feedback action of oestradiol results in increased LH-RH secretion has been shown indirectly by further studies in the human. Malacara et al (1972) and Arimura et al (1974) have demonstrated an increase in peripheral LH-RH concentrations on the day of the midcycle LH peak. Also, by measuring pituitary responses to large (150 ug) and small (10ug) doses of LH-RH, Yen et al (1974c) showed that an oestrogen induced amplification of LH release by the pituitary requires large doses of LH-RH and that the magnitude of acute LH release in response to a small dose of LH-RH is not significantly influenced by increments in oestrogen levels. These findings suggest (although indirectly) that the increase in LH secretion associated with the midcycle surge at least in part depends upon an increased LH-RH output, and that this in turn may be due to the positive feedback action of oestradiol.

1.5 The dynamics of LH release in relation to steroid feedback: Pituitary sensitivity and capacity

It is now evident that pituitary LH secretion varies dramatically according to the nature of the LH-RH stimulus. Early studies measuring pituitary response to synthetic LH-RH used single, relatively large doses of the releasing hormone, and it is only fairly recently that continuous infusion or pulsed delivery of much smaller doses has been performed. These later techniques have revealed several interesting and important characteristics of pituitary LH secretion, and have provided a more complete understanding of the relationship between steroids and the hypothalamic pituitary system. There is evidence to suggest that the interaction between LH-RH and oestradiol may reflect the functional presence of two pools of LH in the pituitary; an immediately releasable pool, and a storage pool. These two pools of LH may help to elucidate the ideas of pituitary "sensitivity" and "capacity" which have been the subject of some recent detailed studies in the human.

Constant stimulation with small amounts of LH-RH can result in a biphasic pattern of LH secretion in normal men (Bremner and Paulson, 1974), in pubertal, but not prepubertal children (Reiter, Duckett and Root, 1975), in normally cycling women (Yen et al, 1975a; Wang, Lasley, Lein and Yen, 1976; Hoff, Lasley, Wang and Yen, 1977) and hypogonadal women (Lasley, Wang and Yen, 1975). These, and other studies, have led to the idea of multiple components of LH release in terms of two functionally separable pools of pituitary LH: - one, immediately releasable, reflecting pituitary sensitivity; the other requiring continued stimulus input, reflecting pituitary

capacity (i.e. the storage pool which includes a component of newly synthesised LH). Thus when LH-RH stimulation is small and brief, LH increments may be a reasonable measure of pituitary sensitivity, whereas estimation of pituitary capacity or reserve will probably require a longer duration of stimulation, achieved either by constant infusion or by pulsed delivery of the releasing hormone, thereby simulating the (assumed) normal hypothalamic input. Measurement of the response to a single large dose of LH-RH primarily measures sensitivity although a variable and indefinable degree of "capacity" may also be involved, and thus it is not possible to obtain an accurate measurement of either component.

The techniques of constant infusion or pulsed delivery of submaximal doses of LH-RH to study the functional capacity of the pituitary gland in the human have been mainly used by Yen and his colleagues. Lasley et al (1975) clearly demonstrated that pituitary response to pulses of LH-RH (10 ug at 2 hourly intervals) was augmented in terms of both sensitivity and reserve by incremental changes in circulating oestradiol levels achieved by daily administration of oestradiol benzoate for 4 days during the early follicular phase. These workers also showed that a brief exposure (4 hrs) to relatively low levels of progesterone at the end of the oestradiol benzoate treatment induced a marked amplification of the oestrogen augmented pituitary sensitivity and reserve. Thus a direct action of progesterone on the gonadotrophs may explain the facilitatory action of progesterone on LH release in oestrogen primed conditions described by several laboratories (e.g. Odell and Swerdloff, 1968; Yen et al, 1975 a).

A similar augmentation of pituitary sensitivity and capacity by oestrogen treatment has been observed with constant infusion of a small dose of LH-RH (0.2 ug/min for 4 hours) (Yen et al, 1975a). Although oestrogen and progesterone can enhance pituitary response to LH-RH in intact women, it has been demonstrated that pituitary sensitivity is greatest in hypogonadal subjects (Lasley et al, 1975; Wang et al, 1976), and that in hypogonadal women oestrogen treatment for 7 days induces an impediment of sensitivity but a marked augmentation of pituitary reserve (Lasley et al 1975). It therefore appears that in an open feedback situation such as is found in hypogonadal subjects (where the hypothalamic pituitary system is influenced primarily by the hypophysiotrophic effect of increased endogenous LH-RH secretion (Seyler and Reichlin, 1973)), there is a large pool of acutely releasable LH with a relatively smaller reserve pool. In this context it is of interest to note that Wang et al (1976) found that increased doses of LH-RH to hypogonadal women were not able to elicit additional LH release, suggesting that pituitary sensitivity is at a maximum. It is probable that the decline of basal LH secretion during oestrogen treatment in hypogonadal or agonadal subjects may be a consequence of a decreased pituitary sensitivity (Yen et al 1974c) concomitant with a reduction of hypothalamic LH-RH secretion (Seyler and Reichlin, 1973). The preferential augmentation of pituitary reserve during this event probably represents the principal action of oestrogen directly on the gonadotrophs and is seen only in the presence of an exogenous LH-RH stimulus (Lasley et al, 1975).

Of relevance to the concept of pituitary sensitivity and capacity is the "self-priming" effect of LH-RH. This effect,

originally demonstrated in the rat by Aiyer, Chiappa and Fink (1974), and since confirmed in humans (Wang et al, 1976; Hoff et al, 1977) appears to be oestrogen dependent. During the high oestrogen phases of the menstrual cycle (late follicular and mid luteal) LH responses to a second pulse of LH-RH (2 hours later) were significantly greater than those following the first pulse of LH-RH, although this pattern of response was not observed during the early follicular (low oestrogen) phase. Thus during high oestrogen phases of the menstrual cycle the first pulse of LH-RH appears to "prime" the pituitary to produce increased responses to subsequent LH-RH pulses.

The physiological significance of oestrogen induced LH-RH self priming, and attenuation and amplification of pituitary sensitivity and capacity by ovarian steroids will be examined in the final section of this review.

1.6 Functional characteristics of the hypothalamic-hypophysial-gonadal system in the regulation of cyclic LH release

The cyclic release of LH to produce a normal menstrual cycle is the result of complex interactions between the ovary, the hypothalamus and the pituitary. It appears that in the human two functional pools of LH are present in all phases of the menstrual cycle and that the comparative pool size and activity is profoundly influenced by ovarian steroid feedback, as well as by the pattern of input of hypothalamic LH-RH (Wang et al, 1976; Hoff et al, 1977).

From the early to the late follicular phase, in synchrony with the rising levels of oestradiol, the size of the 2nd pool

(capacity) is preferentially augmented. A small increase in the 1st pool (sensitivity) activity is not apparent until the late follicular phase when a 5 fold increase in the size of the second pool is also attained. The increase in the 1st pool size seen during the late follicular phase probably results from a rapid activation of the larger 2nd pool to the more readily releasable 1st pool. Although the precise mechanism responsible for this phenomenon is not known, it may be due to an oestrogen induced increase in endogenous LH-RH (Seyler and Reichlin, 1974b)and/or to a development of a self-priming effect of LH-RH at this time (Wang et al, 1976). During the mid luteal phase, and in association with relatively high levels of oestrogen and progesterone, the large 2nd pool is maintained as in the late follicular phase, but the 1st pool is strikingly smaller. A possible explanation for this may be that the pituitary gonadotrophs remain highly sensitive to LH-RH under the combined influence of oestrogen and progesterone, but that the extremely low endogenous release of LH-RH at the mid luteal period obviates the self priming effect of LH-RH. This explanation seems reasonable from the observation that infusion (priming) with exogenous LH-RH during the luteal phase will result in a massive augmentation to subsequent LH-RH stimulation (Hoff et al, 1977).

It seems likely that the oestrogen induced LH-RH self priming may serve to activate the reserve pool and render its LH more readily releasable; this is then revealed as an increase in pituitary sensitivity. This theory is substantiated by the finding that during the days of the mid cycle LH surge, a dramatic reversal of the relative activity of the two pools is observed which is

manifested by an enormous increase in the activity of the first relative to the second pool. In contrast to the other phases of the cycle, the release of LH from the 2nd pool is not sustained and this premature decline in LH release despite continuous LH-RH stimulation appears to be due to pituitary depletion of LH.

It therefore appears that LH-RH not only induces synthesis, storage (2nd pool) and release (1st pool) of LH, but also activates the 2nd pool and renders its LH more readily available (self-priming). These positive influences exerted by LH-RH are amplified by the presence of oestrogen which appears to provide a permissive action of LH-RH, except that oestrogen also functions to impede the LH-RH mediated release of LH. At mid cycle, the increased endogenous LH-RH release, together with the development of oestrogen dependent self priming effect of LH-RH induces a dramatic shifting of LH from the 2nd to the 1st pool, with accelerated LH release by overcoming the impeding action of oestrogen. Mid luteal levels of progesterone do not inhibit the augmented activity of the 2nd pool due to oestrogen, but may in fact greatly amplify the LH-RH induced activation of the 2nd pool, with the enlargement of the smaller 1st pool. The physiological significance of this extra-ordinary enhancement of pituitary responsiveness to LH-RH (after priming) is not clear, for progesterone levels are not found to be significantly elevated before the onset of the LH surge in humans (Johansson and Wide, 1969). However the possibility of progesterone functioning as a secondary signal in the maintenance of the midcycle surge should be considered.

In conclusion, this review has attempted to bring together some of the information relating to the control of LH secretion in primates. It can be seen that the secretion of LH by the pituitary gland is governed by complex interractions between the hypothalamus, the pituitary and the gonads. Although our understanding of the mechanisms regulating LH secretion has increased considerably in the last 10 years, our knowledge is far from complete. The importance of catecholamines is realised, but how and where they act is unclear, and little is known about the role of prostaglandins in the control of LH secretion. Furthermore there is little information on 'short-loop' (where the pituitary influences LH-RH secretion, Kühl and Taubert, 1975) and 'ultra short-loop' (in which LH-RH controls its own secretion, Hyyppa, Motta and Martini, 1971) feedback mechanisms which are thought to exist. These, and many other considerations may form the basis of research into the control of LH secretion in the next 10 years.

1.7 The aims and scope of the study

The importance of the hypothalamic-hypophysial-gonadal system in regulating LH secretion in primates is well established from studies mainly on the rhesus monkey and human. In these species the secretory patterns of hypothalamic hormones, pituitary

gonadotrophins and gonadal steroids have been studied in detail, although their interrelationships are not yet fully understood. Knowledge of the physiology of the hypothalamic-hypophysial-gonadal system in non-human primates may be of value in the understanding of reproductive endocrinology in humans.

This study was undertaken to examine certain aspects of the hypothalamic-hypophysial-gonadal system in the marmoset monkey, with the aim of determining some of the mechanisms involved in controlling the secretion of LH in this species. The marmoset has certain advantages over larger, more conventional primates which make it an attractive alternative as an animal for laboratory research. Detailed knowledge of its reproductive endocrinology is essential in assessing the potential of this species as a possible model for the human. The present study has been designed to provide some of this information, and, where possible, to extend previous observations from studies in other primates.

In recent years there has been considerable interest in developing new forms of fertility control, particularly by methods directed at the hypothalamic-pituitary axis. Techniques for inhibiting LH-RH action have been developed, although detailed study of their effects in primates is required before use in humans can be envisaged. Inhibition of LH-RH is one way in which its action can be studied, and by using the marmoset the potential of this approach in human

fertility control could be examined.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

The animals used in this study were common marmoset monkeys, Callithrix jacchus. They were maintained at the MRC Unit of Reproductive Biology Primate Laboratory at the Bush Estate, Midlothian. Animals used were adults (over 2 years old and 280 gm body weight) either obtained from the wild, more than 18 months before their use in experiments and fully adapted to captive conditions, or born in captivity.

2.2 Management

Marmosets were kept in male-female pairs in aluminium cages (50 x 50 x 75 cm) containing a nest box and two perches. To prevent boredom and to maintain animals in a healthy condition, each pair of marmosets was allowed periodic access to a large exercise cage (150 x 100 x 210 cm). The animals rooms were maintained at a temperature of 24°C (range 21 - 29°C) and 65% relative humidity (range 55 - 70%), and were ventilated at about 10 changes of air per hour. Animals were given natural light with additional illumination between 0500 hours and 1900 hours. Hearn, Lunn, Burden and Pilcher (1975) have published full details of the management of this colony.

2.3 Collection of blood

Blood samples were taken from the femoral vein using an 0.46mm diameter (27 gauge) needle and a heparinised 1ml syringe, and placed immediately on ice. The syringe was sealed with a steriseal cap, centrifuged at 2500 rpm for 20 minutes at 4°C, and plasma stored at -20°C until assayed. Animals were either restrained manually by a handler (Figure 2.1) or in a restraining device that

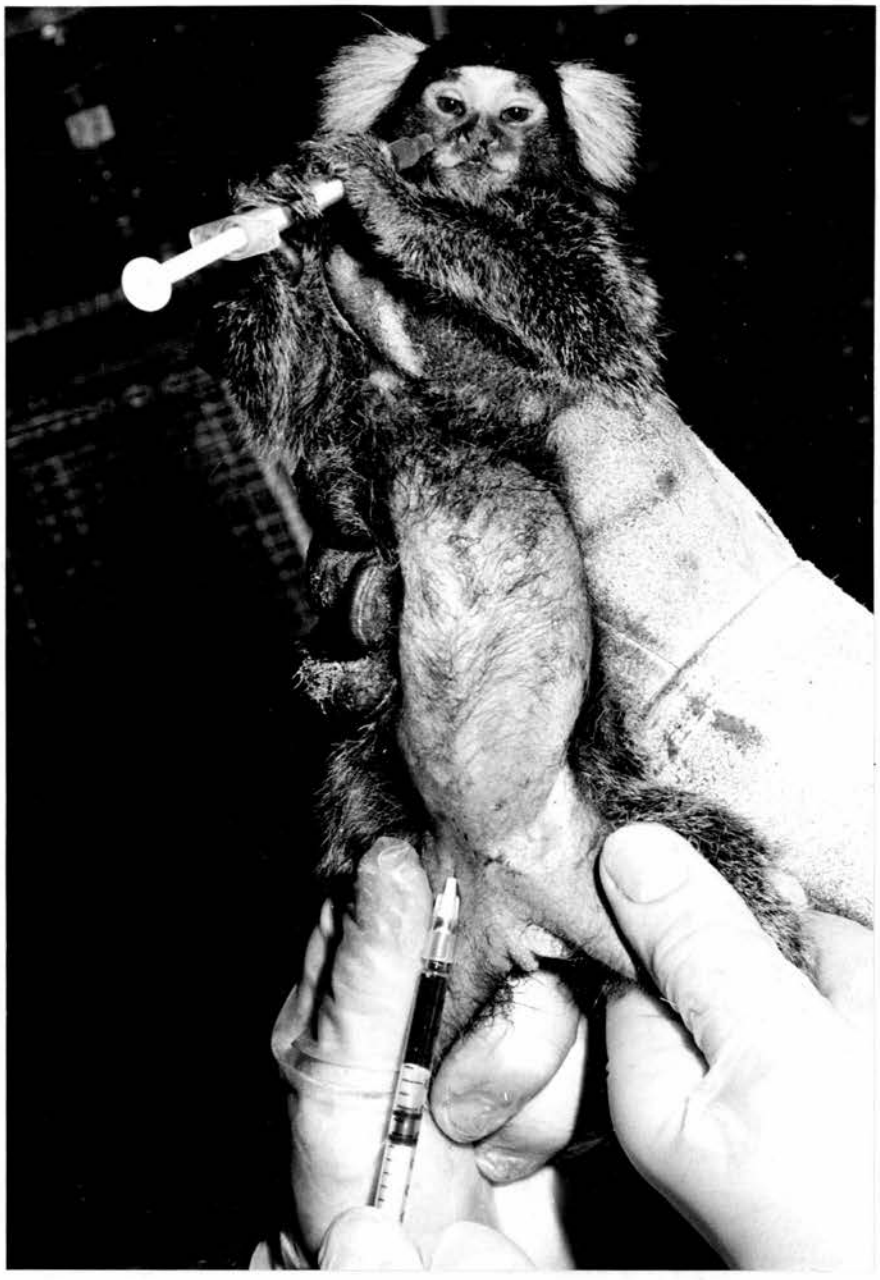


Figure 2.1

Collection of blood from a marmoset, restrained manually by a handler

allowed a single person to manipulate the monkey and collect the blood sample (Hearn, 1977) (Figure 2.2).

When either system was in use, the marmosets remained relaxed and appeared to be under little stress even during serial bleeds. After collecting the blood sample ,the animals were given 0.1 - 0.2 ml of iron syrup ("Fersamal", Glaxo Laboratories Ltd.) as a reward, and for replacement of iron.

2.4 Hormones for injection

The following hormones were used for injection:-

1. Progesterone in arachis oil, 10mg/ml (Organon Laboratories, Ltd.), diluted further as required in arachis oil.
2. Oestradiol benzoate in arachis oil, 1mg/ml (Organon Laboratories, Ltd.), diluted further as required in arachis oil.
3. Testosterone and dihydrotestosterone were obtained from Sigma Ltd., as crystalline preparations, and were dissolved in arachis oil.
4. Luteinising hormone releasing hormone (LH-RH) in saline, 100 ug/ml, was kindly donated by Hoechst Pharmaceuticals and was further diluted in 0.9% saline before use. A freeze-dried preparation of LH-RH (Hoechst Pharmaceuticals) was conjugated to bovine serum albumen (Sigma Chemical Co.) for use as an immunogen.
5. Analogues of LH-RH (D-Phe^{2}Phe^{3}DPhe^{6}LH-RH, and D-Phe^{2}D-Trp^{3} D-Phe^{6}LH-RH) were generously donated by Dr. A.V. Schally, and were dissolved in 20% propylene glycol (in 0.9% saline).

2.5 Administration of hormones

All steroids, injected in 0.1ml of oil, were given subcutaneously in the ventrolateral region of the abdomen.

Figure 2.2

A marmoset in the restraining device used for collecting blood

LH-RH and LH-RH analogues were given as rapid intramuscular injections in 0.1ml of 0.9% saline or 20% propylene glycol respectively.

2.6 Steroid implants

25mg and 50mg crystalline progesterone implants were obtained from Organon Laboratories Ltd. Oestradiol-17β implants were prepared using a modification of the technique described by Dzuik and Cook (1966):-

One end of a 14mm length of Silastic Medical Grade tubing (1.96mm o.d. x 1.47mm i.d., Dow Corning Corporation) was sealed with a 2mm plug of silastic medical grade adhesive (Silicone type A, Dow Corning). The length of tubing, in a small plastic dish, was carefully lowered into a kilner jar containing about 15ml of distilled water. After the lid of the kilner jar had been tightly closed and the inlet valves clamped, the jar was placed in a water bath and incubated at 37°C for 24 hours. This procedure creates a water saturated atmosphere inside the jar and hardens the adhesive. The silastic tube was then removed and packed with crystalline oestradiol-17β (Sigma Ltd.), leaving a 2mm gap at the open end. The tube was then sealed and incubated as described above. The implants produced in this way contained a 1.0cm long column of oestradiol-17β .

To test the rate of release of oestradiol-17β , six implants were incubated in vitro as follows:-

Each implant was placed in a glass jar containing 50ml phosphate gelatin buffer (see page 69) and incubated (with constant shaking) in a water bath at 37°C for 3 days. Every 24 hours the incubation medium was changed and a small aliquot diluted in buffer

and assayed for oestradiol-17β (see page 77). The concentration of oestradiol (pg/sample) was then used to calculate the approximate amount of oestradiol-17β released into the medium in 24 hours. Mean (with range) release rates for each of the three days were 17.6 (12.1-22.6), 13.7 (10.4-18.6) and 13.5 (10.1-16.4) ug oestradiol-17β /day.

2.7 Surgery

Surgery was performed under aseptic conditions. All animals for surgery were anaesthetised with an intramuscular injection of 0.5ml "Saffan" (18mg/kg) (Alphaxalone 0.9% w/v, Alphadolone acetate 0.3% w/v; Glaxo Laboratories Ltd.), and received an intramuscular injection of 0.2ml penidural (Fortified Injection Veterinary - John Wyeth and Brother). The dose of anaesthetic was sufficient to maintain animals under controlled, deep anaesthesia for a period of about 1 hour. When the anaesthetic had taken effect the animals were shaved in the appropriate region, the skin disinfected with Hibiscrub (I.C.I. Pharmaceuticals Ltd.), and the surrounding area covered with surgical dressing.

2.8 Surgical procedures

In the course of this study the following surgical procedures were performed:-

1. Orchidectomy.
2. Laparotomy.
3. Ovariectomy.
4. Insertion of implants.

1. Orchidectomy

The base of the testis was clamped to project the testis forward tightly against the scrotum. A 5-8mm incision was made in the scrotal skin with a scalpel blade. The testis and epididymis was gently squeezed out through the incision using forceps. The spermatic cord was ligated in two places (about 2-3mm apart) about 10mm along its length from the epididymis, and the testis and epididymis removed after cutting between the ligatures. A thin film of penicillin powder was applied to the wound and the incision in the scrotal sac was closed with one or two stitches. 3/0 Chromic sutures with a 16mm cutting needle (Ethicon Ltd.) were used.

2. Laparotomy

A 10-15mm vertical incision was made through the skin in the ventral region of the abdomen, immediately to the right of the midline. A similar incision was made through the muscle layer, taking care to leave the peritoneum intact. The abdominal cavity was then exposed by cutting through the peritoneum using scissors. The ovaries and uterus were measured using calipers and their appearance noted. The incisions in the peritoneum and muscle layer were closed with a continuous stitch (4/0 Chromic suture) and the tissue carefully aligned. After the application of penicillin powder the incision in the skin was closed with a continuous subcuticular purse stitch and then with two or three loose surface stitches.

3. Ovariectomy

The procedure was as described for laparotomy except that the ovarian pedicles, arteries and veins were ligated with 3/0 Chromic suture and the ovaries removed after cutting through the attachments with scissors.

4. Insertion of implants

A 3-5mm incision was made through the skin and connective tissue in the ventrolateral region of the abdomen. The incision was held open with forceps while the implant was introduced with a second pair of forceps to lie at least 15mm below the incision. Penicillin powder was applied and the incision closed with a single stitch.

2.9 Immunisation against LH-RH

1. Preparation of immunogen

LH-RH was conjugated to bovine serum albumen (BSA, Sigma Chemical Co. Ltd.) using 1-ethyl-3(3-dimethyl-amino-propyl-carbodiimide (Sigma) according to the method of Fraser, Gunn, Jeffcoate and Holland (1974b). For each animal to be immunised 1mg LH-RH, 1mg BSA and 10mg carbodiimide were used.

Method

(a) LH-RH was weighed into a 5ml bottle, 0.5ml distilled water and BSA were added and the reagents mixed for 1-2 mins.

(b) Carbodiimide was weighed into a separate container, dissolved in 0.25ml distilled water and added to the LH-RH/BSA solution. The reagents were mixed gently for 1 minute.

(c) After leaving the mixture at room temperature in the dark for at least 12 hours, the contents were transferred to a dialysis sac (about 15cm long, Viscing Tubing; Scientific Instruments Centre Ltd.), and the container washed with 1ml of distilled water.

(d) The contents of the tubing were dialysed against distilled water at 4°C for 48 hours and against 0.15M saline at 4°C for a further 24 hours.

(e) The contents of the dialysis sac were transferred to a 10ml container and made up to the required volume with 0.15M saline.

To enhance the immunological response to the LH-RH immunogen, the conjugate was emulsified with Freunds complete adjuvant (FCA) (Difco Laboratories) for primary immunisations, and with Freunds incomplete adjuvant (FIA; Difco Laboratories) for booster injections. The procedure was as follows:-

(a) Equal volumes of adjuvant and antigen solution were added in a universal container (30 ml) to give a total volume sufficient to allow 1.0ml of emulsion to be given to each animal (allowing 10-20% loss during emulsification).

(b) The mixture was emulsified with an electric homogeniser at medium speed for 2-3 minutes, or until the required viscocity had been obtained.

(c) The emulsion was tested by allowing a few drops to fall into a beaker of cold water. A sufficiently emulsified preparation remained as a white drop on the surface.

An emulsion of equal volumes of BSA in saline and FCA was used for control immunisations.

2. Immunisation

Marmosets were anaesthetised and 1ml of emulsion was distributed in 8-10 intradermal injections along the dorso-ventral region. A single intramuscular injection of 0.25ml Bordetella pertussis vaccine (Burroughs Wellcome and Co., London) to act as an additional adjuvant was then administered.

2.10 Radioimmunoassay for Luteinising Hormone

Plasma luteinising hormone was measured using a double antibody radioimmunoassay which is a modification of the assay for rat LH described by Welschen, Osman, Dullaart, De Greef, Uilenbroek and De Jong (1975). The system which was finally adapted to measure LH in marmoset plasma utilises NIAMDD-rat LH I-1 for iodination, NIAMDD-rat LH RP-1 as standard, and anti-ovine LH 610 V as antiserum. The antiserum was raised in a rabbit by immunisation with NIH-LH S17.

2.10.a Buffers

1. Phosphate buffered saline (PBS) (0.01M; pH 7.8) was made from stock solutions of 0.1M disodium hydrogen orthophosphate (A) and 0.1M sodium dihydrogen orthophosphate (B). Sodium chloride (36 gm), 91.6ml of A and 8.1ml of B were made up to 1 litres with distilled water. Sodium thiomersalate (Hopkins and Williams, Ltd.) (0.01gm) was added as preservative.

2. Phosphate buffered saline (PBS) (0.05M; pH 7.8): This buffer was prepared exactly as above except that the solution was made up to 800ml.

3. Phosphate buffered saline plus bovine serum albumen (PBS + BSA): BSA was added to 0.01M PBS to give a concentration of 1gm per 100ml (1% BSA).

4. "Special buffer"

3.72gm ethylenediaminetetra-acetic acid (EDTA, Sigma) were dissolved in 1 litre of 0.01M phosphate buffered saline. The pH of this solution was adjusted to 7.5 using 3N sodium hydroxide. Normal rabbit serum (6.7ml) (NRS; Wellcome Reagents, Ltd.) and 2gm BSA were added.

5. Barbitone buffer (0.12M; pH 8.5): Diethyl barbituric acid (110gm) was dissolved in 4.5 litres of distilled water, and 19gm of sodium hydroxide in one litre of deionised water was added to the main solution and stirred for 2 hours. The solution was then made up to 5 litres with distilled water and stirred for 24 hours.

6. Barbitone buffer plus BSA: 5gm BSA were dissolved in 100ml Barbitone buffer (i.e. 5% BSA).

Unless otherwise stated all reagents were of Analar grade from BDH Chemicals Ltd. All buffers were stored at 4°C.

2.10.b Iodination of Rat LH

Rat LH preparation NIAMDD-rat LH I-1 was labelled with $Na^{125}I$ (Radiochemical Centre, Amersham) by a modification of the chloramine-T method of Greenwood, Hunter and Glover (1963). Two micrograms of hormone were reacted with 0.5-1.0m Ci $Na^{125}I$ and 50ug chloramine T in a plastic tube (11 x 63mm, Sarstedt). The chloramine T was made up immediately before use to a concentration of 5mg/ml in 0.05M phosphate buffer; 10ul of this solution was used in the reaction. The reaction was stopped after 30 seconds

by adding 125 ug sodium metabisulphite in 0.5ml 0.05M phosphate buffer. Potassium iodide (10mg) was then added in 0.5ml 0.12M barbitone buffer (pH 8.6). Labelled LH was isolated from the reaction mixture by adsorption chromatography on a small column (1 x 5cm) of Whatman CF II grade cellulose (Whatman Phamaceuticals). The reaction mixture was applied to the column which was then washed with 30ml 0.12M barbitone buffer. The ^{125}I labelled LH was eluted from the column by passing through 10ml of barbitone buffer containing 5% BSA, and collecting 1ml fractions. The most immunoreactive fractions were pooled, and no further purification was necessary.

2.10.c Standards

Standard LH solutions were prepared from 50 ul aliquots (10ug hormone) stored at -20°C by diluting to a total volume of 12.5ml in PBS + 1% BSA buffer. This gave a concentration of 800 ng/ml, and standard solutions were prepared as doubling dilutions of this solution. For each assay standards were aliquoted in duplicate so that tubes contained 160 - 0.6 ng in a volume of 200 ul.

2.10.d Antiserum

Anti-ovine LH 610V was stored at -20°C in 50ul aliquots at a dilution of 1:10. This was diluted to 1:1000 with 0.01M PBS and stored at 4°C. For each assay this solution was diluted further in special buffer to give an initial concentration of 1:70000. This dilution of antiserum bound 21-30% of the labelled tracer (n=15).

2.10.e Assay procedure

All samples were assayed in duplicate using plastic 63 x 11 mm tubes. The protocol of each assay included:-

1. Total counts tubes (TC): ^{125}I-LH (100ul).
2. Non-specific binding tubes (NSB): special buffer (100ul); PBS + 1% BSA (200ul); ^{125}I-LH (100ul).
3. Total bound tubes (TB): PBS + 1% BSA (200ul); antiserum (100ul); ^{125}I-LH (100ul)
4. Standards: standard LH (200ul); antiserum (100ul); ^{125}I-LH (100ul)
5. Unknowns and quality controls: plasma (50ul); PBS + 1%BSA (150ul); antiserum (100ul); ^{125}I-LH (100ul).

Standards were dispensed in a volume of 200ul PBS + 1% BSA buffer. Test samples were dispensed in 50ul aliquots and made up to 200ul with PBS + 1% BSA buffer. Antiserum (100ul) was added and the contents of each tube mixed and incubated at 4°C for 3 days. ^{125}I-labelled LH (approximately 10000cpm in 100ul PBS + 1% BSA buffer) was added to all tubes which were mixed and incubated at 4°C for a further 2 days. Separation of antibody bound and free hormone was achieved by adding 200ul of donkey anti-rabbit gamma globulin (1:30 v/v, in 0.01M PBS) (Burroughs Wellcome, RD 17) and incubation was continued at 4°C for 12 hours. The unbound radioactivity was diluted with 1ml 0.01M PBS and the tubes centrifuged at 4°C for 30 minutes at 2500 rpm. The supernatants were discarded, the tubes dried with tissue paper, and the antibody-bound ^{125}I-labelled LH in the precipitate was measured in an automatic gamma spectrometer (Wallac Decem - GT2).

Eppendorf pipettes with disposable tips were used throughout the assay for dispensing the standards and plasma samples. Buffer was added to assay tubes with a semi-automatic dispenser (Lumix: Chem. Lab. Instruments Ltd.), and repettes (Jencons) were used to add iodinated hormone and antiserum to the tubes.

2.10.f Precision

The precision of the assay was assessed by repeated assay of two pools of marmoset plasma from male and female marmosets following LH-RH administration. One pool was used to measure intra-assay variation and had a value of 80.1 $\pm$ 2.86 ng/ml, with a coefficient of variation of 3.57% (n=12). A second plasma pool was used for determining inter-assay variation and had a value of 87·24 $\pm$ 6.49 ng/ml, with a coefficient of variation of 7.4% (n=15).

2.10.g Sensitivity

The limit of detection of the assay (B/BO = 90%) ranged from 0.7 - 0.9 ng/tube. Since the dilutions of marmoset plasma and rat standard became non-parallel at above 87% B/Bo (see page 62), a working sensitivity of the assay of 1ng/tube was adopted. With a 50ul plasma sample the detection limit was therefore 20ng/ml LH RP-1 equivalent.

2.10.h Calculations

A typically sigmoid standard curve was obtained using a Y axis of B/Bo (where B = counts in the standard tubes - counts in the NSB tubes; and Bo = counts in the TB tubes - counts in the NSB tubes), and an X axis of the nanogram values of the standards on a

log scale at a dose interval of two. Figure 2.3 shows a typical specimen curve. The curve was drawn to pass through the mean value of each dose of standard. Since dilutions of marmoset plasma and NIAMDD rat LH RP-1 became non parallel above 87% B/Bo and below 31% B/Bo,LH concentrations were calculated only for samples falling within these limits.

2.11 Validation of the assay for marmoset LH

2.11.a Methods

1. Specificity of the LH assay

200ul of doubling dilutions of rat LH I-1, human LH (hLH-Stockell-Hartree IRC_2), human FSH (hFSH - Butt CPDS 15), human TSH (hTSH - Stockell-Hartree De 32-3), ovine LH (oLH-S10), and bovine FSH (bFSH-CH-1-76) were subjected to the same assay procedure as described above. Since no purified marmoset pituitary hormone preparations are available, the antiserum was tested for cross reaction with TSH by measuring the response to an intramuscular injection of 20ug thyrotrophin releasing hormone (TRH; Roche). Plasma obtained from blood samples taken at various times (0, 10, 30 and 50 minutes) after the injection were measured in the LH assay.

2. Measurement of LH in plasma

Physiological validation of the assay was obtained by measuring (1) LH concentrations after LH-RH administration to intact and LH-RH immunised male marmosets; (2) the LH response to gonadectomy (see Chapter 5).

The parallelism between dose response curves for rat LH RP-1 standard and dilutions of marmoset crude pituitary extract

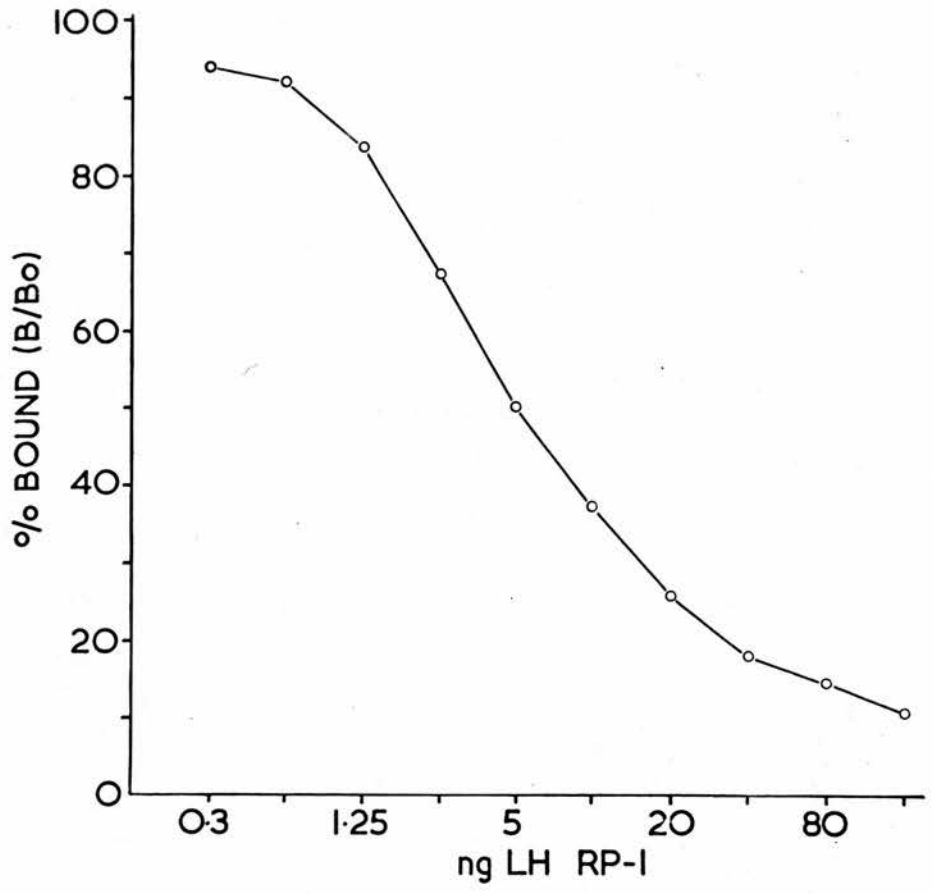


Figure 2.3

Standard curve for LH

and plasma samples containing endogenous marmoset LH from LH-RH treated and LH-RH immunised marmosets was assessed to validate the assay for the measurement of LH in marmoset plasma.

2.11.b Results

1. Specificity of the LH assay

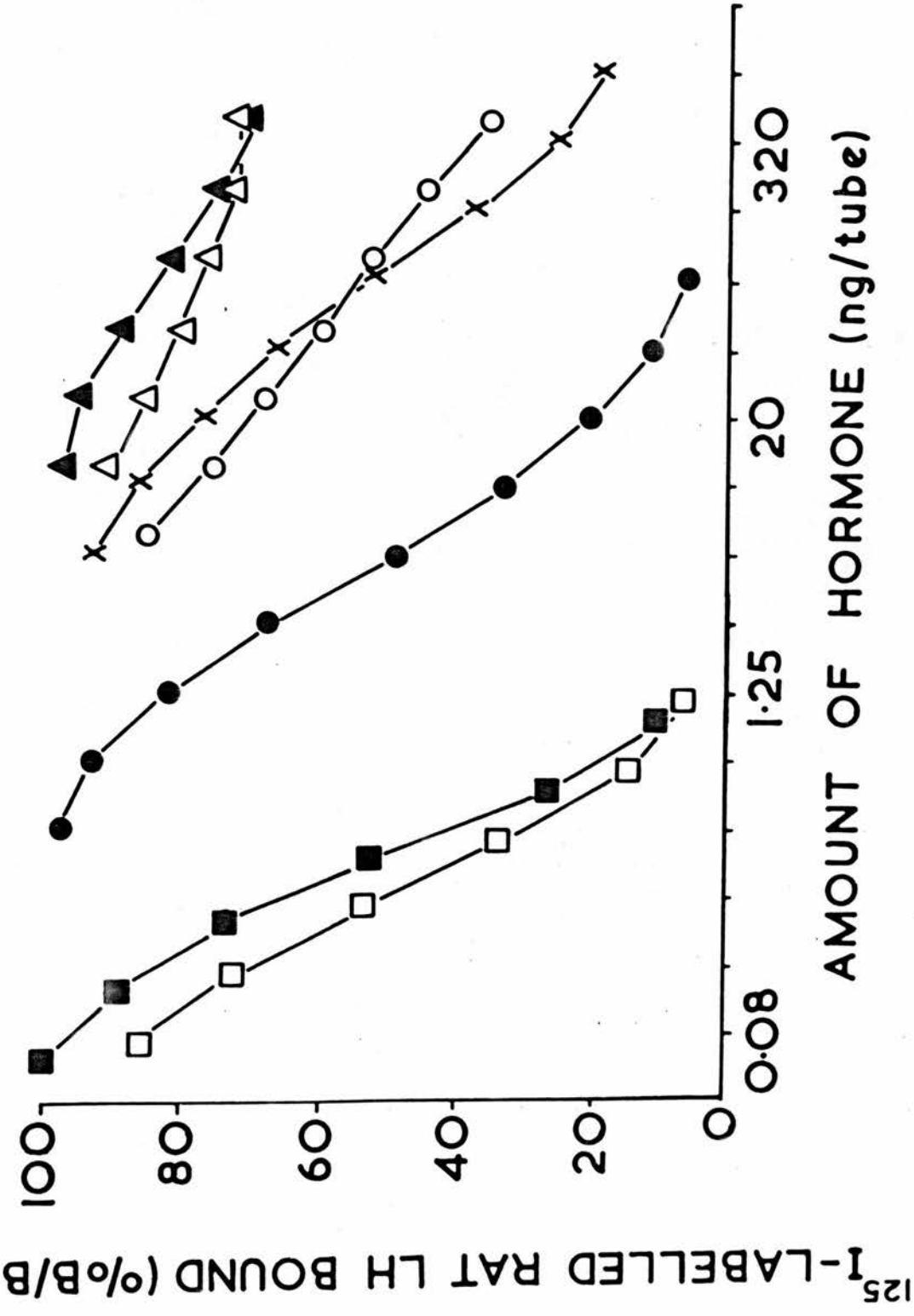
Figure 2.4 shows the cross reactivity of all the standard hormones investigated in the heterologous LH assay. The slope of the dose response curve using oLH-S10 standard was slightly greater than in the system which used an LH standard of rat origin (NIAMDD rat LH RP-1). The highly purified iodination grade NIAMDD rat LH I-1 preparation was used to assess the cross reactivity of the various hormones tested, and was given a potency value of 100%. Cross reaction was calculated as the amount of hormone (w/w) giving 50% inhibition of binding. NIAMDD rat LH RP-1 gave a cross reaction of 3% while cross reactions with bFSH-CH-1-76 and NIAMDD rat FSH I-1 were 0.17% and $< 0.3\%$ respectively (cross reaction with NIAMDD rat FSH I-1 was measured by Welschen et al., 1975). Although all human preparations gave non parallel inhibition curves, the antiserum clearly had a much higher affinity for hLH-1RC2 than for either h FSH-CPDS 15 or h TSH De 32-3, both of which showed no significant inhibition of binding ($< 0.05\%$). There was no marked increase in the concentration of plasma LH after administration of TRH (Fig 2.5).

2. Measurement of LH in marmoset plasma

Serial dilutions of marmoset plasma and a marmoset crude pituitary extract were parallel to the inhibition curve obtained with NIAMDD rat LH RP-1 standard (Fig. 2.6) over the range 31% B/Bo to 87% B/Bo (no significant departure from parallelism, $p > 0.05$, ANOVAR).

Figure 2.4

Cross-reaction of ovine, bovine, rat and human pituitary hormone preparations in the heterologous LH assay. ● , rat LH (NIAMDD rat LH RP-1); □ , rat LH (NIAMDD LH I-1; ■, ovine LH (oLH-S10); x, bovine FSH (bFSH CH-1-76); O , human LH (hLH Stockell-Hartree IRC2); Δ, human TSH (hTSH Stockell-Hartree De 32-3); ▲, human FSH (hFSH ButtcPDS15).



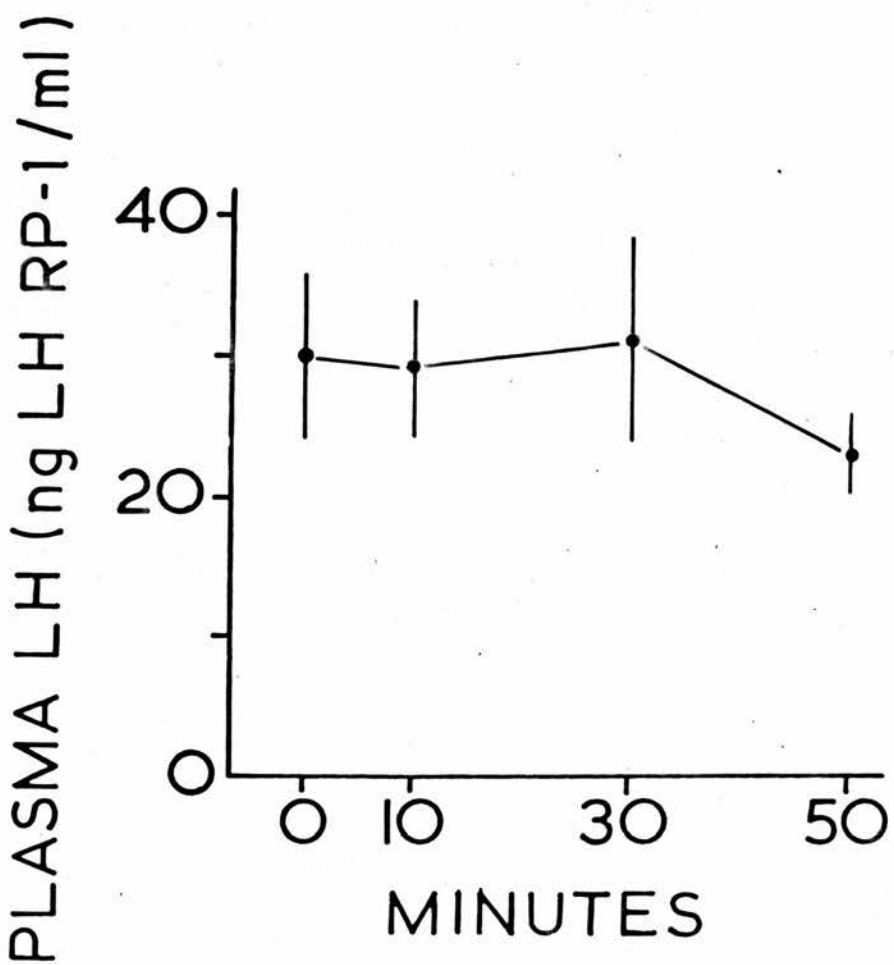


Figure 2.5

Mean (± S.E.M.) LH concentrations in male marmosets (n=3) in response to 20ug TRH

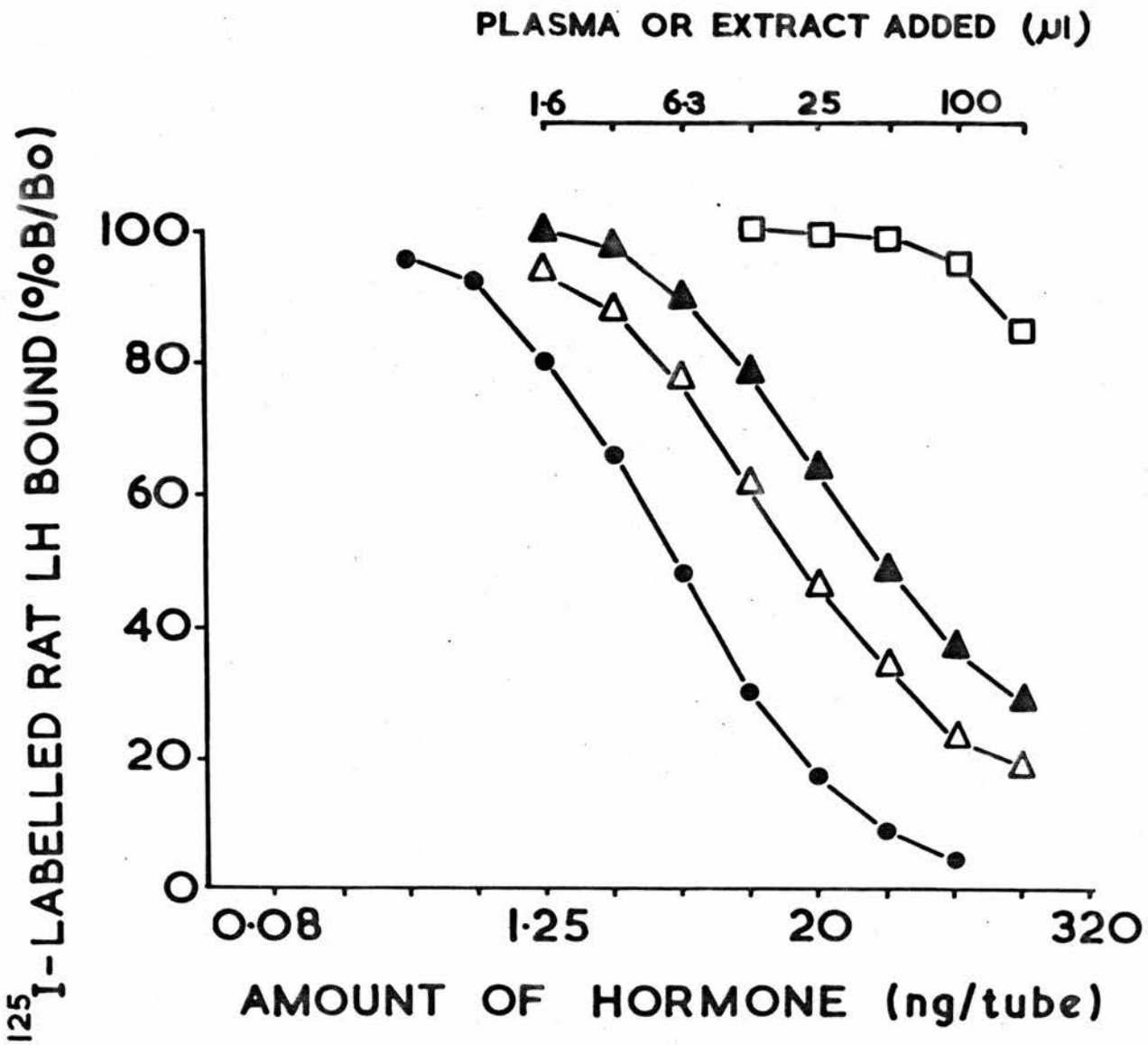


Figure 2.6

Inhibition curves for NIAMDD rat LH RP-1 (●), pooled plasma from LH-RH treated marmosets (△) and LH-RH immunised marmosets (□), and a marmoset crude pituitary extract (▲).

Although parallelism was not found at the extremities of the curves all plasma samples measured in this study fell within the limits of parallelism quoted. There was no significant departure from parallelism ($p > 0.2$, ANOVAR) between serial dilutions of marmoset plasma and the marmoset pituitary extract over the complete range of dilutions. The non-parallelism above 87% B/Bo and below 31% B/Bo seen when comparing marmoset plasma with NIAMDD rat LH RP-1 standard is therefore a feature of the hormone and is not due to a plasma effect. Fifty microlitres of marmoset plasma (the volume used in the assay) showed no non-specific interference. The slight inhibition of binding produced by 200 ul of plasma was probably caused by low levels of residual LH which can occur in LH-RH immunised animals.

Mean ($\pm$ S.E.) LH concentrations before and 30 minutes after LH-RH administration to normal male marmosets were 29.1 $\pm$ 2.5 ng/ml and 102.1 $\pm$ 6.4 ng/ml respectively (n = 15) (Fig. 2.7). The change in the concentration of LH in the plasma (ΔLH) during this period was therefore 73.0 ng/ml. Immunisation against LH-RH inhibited LH release in response to an LH-RH injection. Pre-treatment levels in immunised males were no longer detectable (< 20ng/ml) and mean ($\pm$S.E.) concentration 30 minutes following the LH-RH injection was 25.7 $\pm$ 1.7 ng/ml (n = 3). As pre-injection LH levels were undetectable in immunised animals, it was not possible to obtain a value for ΔLH. Nevertheless it is clear that in animals immunised against LH-RH, circulating LH levels are depressed, and the pituitary response to exogenous LH-RH stimulation is inhibited.

Note

Although absolute validation of the heterologous assay for marmoset LH must await the purification of marmoset pituitary hormones,

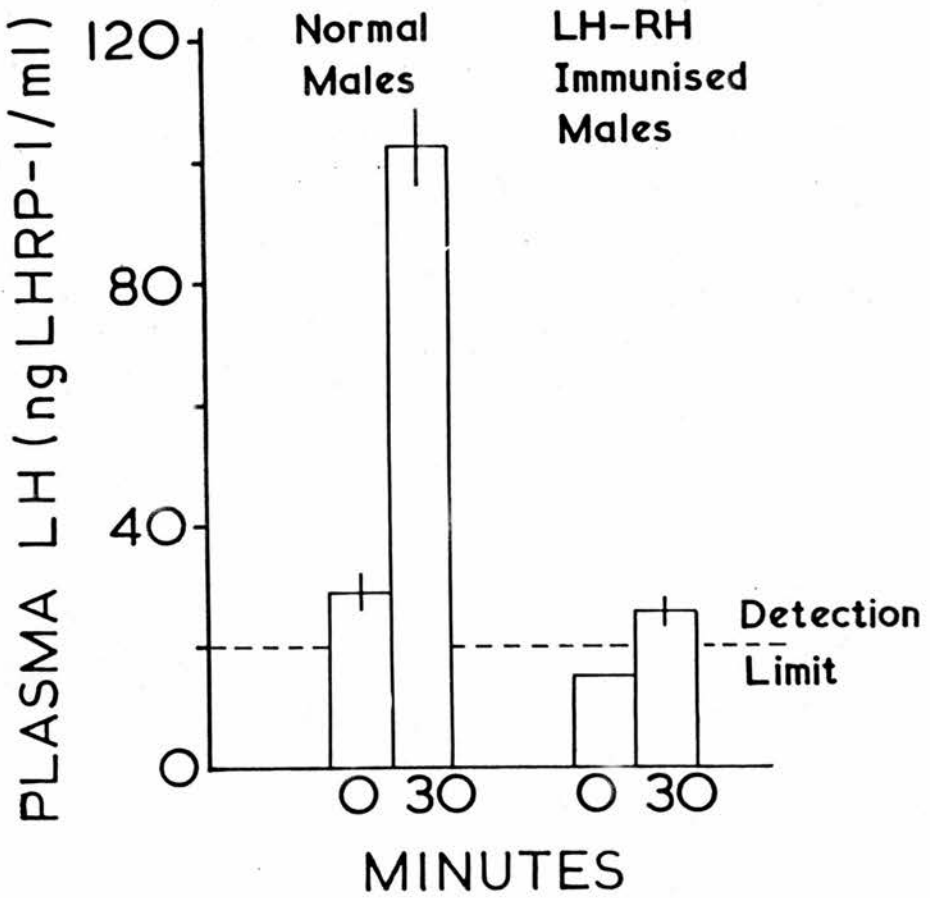


Figure 2.7

Plasma LH concentrations (±S.E.M.) immediately before and 30min after LH-RH administration (4ug) to normal and LH-RH immunised males

the data obtained here indicate that the assay measures marmoset LH. Comparison of dilutions of marmoset plasma and crude marmoset pituitary extract with NIAMDD rat LH RP-1 shows that the assay measures marmoset LH in plasma. The slight inhibition of binding obtained with hTSH DE 32-3 is probably due to contamination of this preparation with LH. Although it is not anticipated that marmoset TSH and hTSH DE 32-3 would show immunochemical similarity, the data relating hTSH and hLH are relevant in showing that the LH assay described here clearly has a much higher affinity for hLH than for hTSH. Since there was no significant increase in the concentration of LH in the plasma of marmosets after administration of TRH, it is assumed that the LH assay is not measuring significant amounts of marmoset TSH (providing TRH does release TSH in the marmoset). The data on hTSH DE 32-3 and the administration of TRH therefore provide indirect evidence that significant cross reaction with TSH in the heterologous LH assay is unlikely.

Physiological validation of the assay (response to administration of LH-RH and gonadectomy) shows that the heterologous assay effectively measures marmoset gonadotrophins, but it does not give any indication of how much FSH is being measured. Cross reaction studies with FSH preparations of human, rat and bovine origin showed a maximum cross-reaction of $<0.3\%$ with NIAMDD rat FSH I-1. Since this assay uses a rat LH tracer, it is unlikely that the cross-reactivity of FSH from any other species will be greater than that of rat FSH. Thus, although a precise determination of the degree of cross-reaction with marmoset FSH cannot be made at present, it is reasonable to assume that it will not exceed 0.3%. These results indicate that this heterologous LH assay provides a reliable and precise method for

measuring plasma LH concentration in the marmoset monkey, although until purified marmoset LH becomes available the accuracy of the assay cannot be determined.

2.12 Radioimmunoassay for Progesterone

Plasma progesterone was measured by a radioimmunoassay method similar to that described by Scaramuzzi, Corker, Young and Baird (1975). The system utilises an antiserum (number 343(7)) (kindly provided by Dr. K. Dighe) which was raised in a rabbit against progesterone-11α-hemisuccinate-bovine serum albumen conjugate. The specificity of the antiserum has been previously tested by Dighe and Hunter (1974).

2.12.a Reagents

Unless stated otherwise, all reagents used were analar grade from BDH.

Assay buffer: 0.05M phosphate gelatin buffer.
45gm of sodium chloride, 43gm of disodium hydrogen orthophosphate, 34gm of sodium dihydrogen orthophosphate and 5gm of gelatin were dissolved in 5 litres of distilled water. Sodium thiomersalate (0.001% $^{w}/_{v}$) was added as a preservative.

Solvents: Analar grade petroleum ether (B.P. 40-60°C), methanol and Aristar grade ethanol were used.

Stripping agents: Activated charcoal Norit A was obtained from Sigma, and dextran T 70 was supplied by Pharmacia Fine Chemicals.

Steroids: Non radioactive progesterone was obtained from Sigma and accurately weighed amounts were dissolved in ethanol and stored at 4°C.

Radioactive progesterone (1,2,6,7-^{3}H-progesterone, approximately 329μCi/μg; Radiochemical Centre, Amersham) was diluted to a concentration of 10 uCi/ml in ethanol and stored at 4°C.

Scintillation fluid: This was prepared by adding 10gm of 2,5-diphenyl-oxazole (PPO; Koch-light) and 750mg of p-tris-(2-(2phenyl-oxazolyl))-benzene (POPOP; Koch-Light) to 2.5 litres of toluene (analytical grade; Koch-Light) . 1.25 litres of Triton X-100 (analytical grade; Koch-Light) were also added and the mixture stirred until a homogenous solution was obtained. 10ml of this fluid were added to each scintillation vial (plastic disposable, New England Nuclear) before counting, using a semi automatic dispenser (Zipette: Jencons).

Disposable glass tubes and eppendorf pipettes with disposable plastic tips were used throughout the assay. The antiserum and tracer were dispensed with 2ml capacity repettes (Jencons) and petroleum ether added using a semi-automatic dispenser (Lumix).

2.12.b Assay procedure

1. Extraction of progesterone from plasma

20ul or 50ul aliquots of plasma were pipetted into glass extraction tubes (75 x 15mm; Gallenkamp) and phosphate gelatin buffer added to produce a total volume of 100ul. 1.0ml of petroleum ether (distilled not more than 24 hours previously) was added and the tubes mixed vigorously in a multivortex mechanical shaker (Baird and Tatlock) for three minutes. When the aqueous and solvent phases had separated out, the aqueous phase was frozen quickly by placing the tubes into methanol containing dry ice. The solvent phase was then decanted into 75 x 12mm glass tubes (Kimble). These tubes were placed in a heated block (Driblock DB3, Tecam) and the solvent evaporated to dryness under a regulated flow of nitrogen.

2. Radioimmunoassay

The dried residue from the extraction procedure was redissolved in 0.3ml of buffer. The contents of the tubes were mixed thoroughly and left to stand at room temperature for at least one hour. The contents of the tubes were mixed for a second time, after which duplicate aliquots of 100 ul of the solution were transferred to glass assay tubes (75 x 10mm; Kimble). 50ul aliquots were transferred directly to counting vials for recovery determination (see below).

For each assay a standard curve was constructed using progesterone standards which had been dissolved in ethanol and diluted in buffer. Standards were serially diluted and aliquoted in duplicate so that tubes contained 31.25 - 1000 ug of progesterone in a volume of 100ul. 100ul of antiserum in buffer (initial dilution 1:10000) and 100ul of 1,2,6,7-^{3}H-progesterone in buffer ($\sim$ 50pg) were added to all tubes containing standards and unknowns, to give a final incubation volume of 300ul. In addition, duplicate sets of total counts (TC), non-specific binding (NSB) and total bound (TB) tubes were set up as follows:-

TC: ^{3}H-progesterone (100ul)

NSB: ^{3}H-progesterone (100ul) + buffer (200ul)

TB: ^{3}H-progesterone (100ul) + buffer (100ul) + antiserum (100ul).

Tubes were incubated overnight at 4°C and then placed on ice while a suspension of dextran coated charcoal in buffer was prepared (25mg dextran and 250mg charcoal per 100mls of buffer). 1.0ml of this suspension (kept on ice and continuously stirred) was added to all tubes, except the TC tubes which received 1.0ml buffer. The tubes were mixed and left on ice for 15 minutes. All tubes were

centrifuged at 2500 rpm for 10 minutes at 4°C, and the supernatants immediately decanted into counting vials. Following the addition of 10mls of scintillation fluid, the vials were allowed to equilibrate in a cooled (4°C) scintillation counter (Packard, Model 3375) for 30-60 minutes before counting.

3. Recovery

The recovery of hormone from plasma was calculated for each sample during the first six assays. Thereafter, recoveries were determined for 6 aliquots of plasma from samples included in the assay. A mean recovery was then applied to all samples in the assay. In the initial six assays mean recoveries ranged from 64-75% and the coefficient of variation for recovery between samples within an assay was 9.76%.

The amount of progesterone extracted from plasma was estimated by adding 20ul of 1,2,6,7-^{3}H-progesterone in ethanol ($\sim$ 2000 cpm) to the plasma/buffer mixture (100ul) in 75 x 15mm glass extraction tubes. Extraction and evaporation to dryness were carried out as described for the assay. Following the addition of 300ul of buffer to the residue, the tubes were mixed and left to stand at room temperature. 50ul aliquots of this solution were then transferred to scintillation vials which were counted as before. Two 20ul aliquots of the tracer (total counts) were also counted.

2.12.c Inter-assay variation

This was determined by repeated assay of duplicate aliquots from a pool of human plasma (Quality controls). A mean ($\pm$S.E.M.) value of 12.4 $\pm$ 0.5 ng/ml was obtained with a coefficient of variation of 11.2% (9 assays).

2.12.d Calculations

A program for the construction of a standard curve and the determination of the amounts of progesterone in plasma samples was written for a desk computer (9821A Calculator: Hewlett Packard) by Mr. R.M. Sharpe. The program was written to construct a straight line from the typically sigmoid standard curve. A standard curve was calculated using a Y axis of the logit transformation of B/Bo, and an X axis of the pg values of the standards on a log scale at a dose interval of 2. Values for logit B/Bo versus dose of standard were plotted by the computer. The calculation of a standard curve omitted the points > 90% of Bo and < 10% of Bo, and a straight line of best fit for the data points was drawn. Progesterone concentrations were calculated by the computer as pg/tube. The results were then corrected to give ng/ml plasma. A typical standard curve obtained by this procedure is shown in Figure 2.8.

2.13 Radioimmunoassay for Testosterone

The radioimmunoassay for testosterone is similar to that described for progesterone, and full details of the methodology and specificity of the assay have been described by Corker and Davidson (1977). The antiserum to testosterone (E.O.I., supplied by Dr. S.A. Tillson, Aliza Corporation, Palo Alto, U.S.A.) was raised in a goat immunised with testosterone-3-oxime coupled to bovine serum albumen. Gross reactions of other steroids tested included: 5α-dihydrotestosterone (25%), oestradiol-17β (0.20%) and androstenedione (0.08%).

The assay for testosterone differs from that for progesterone in the following ways:-

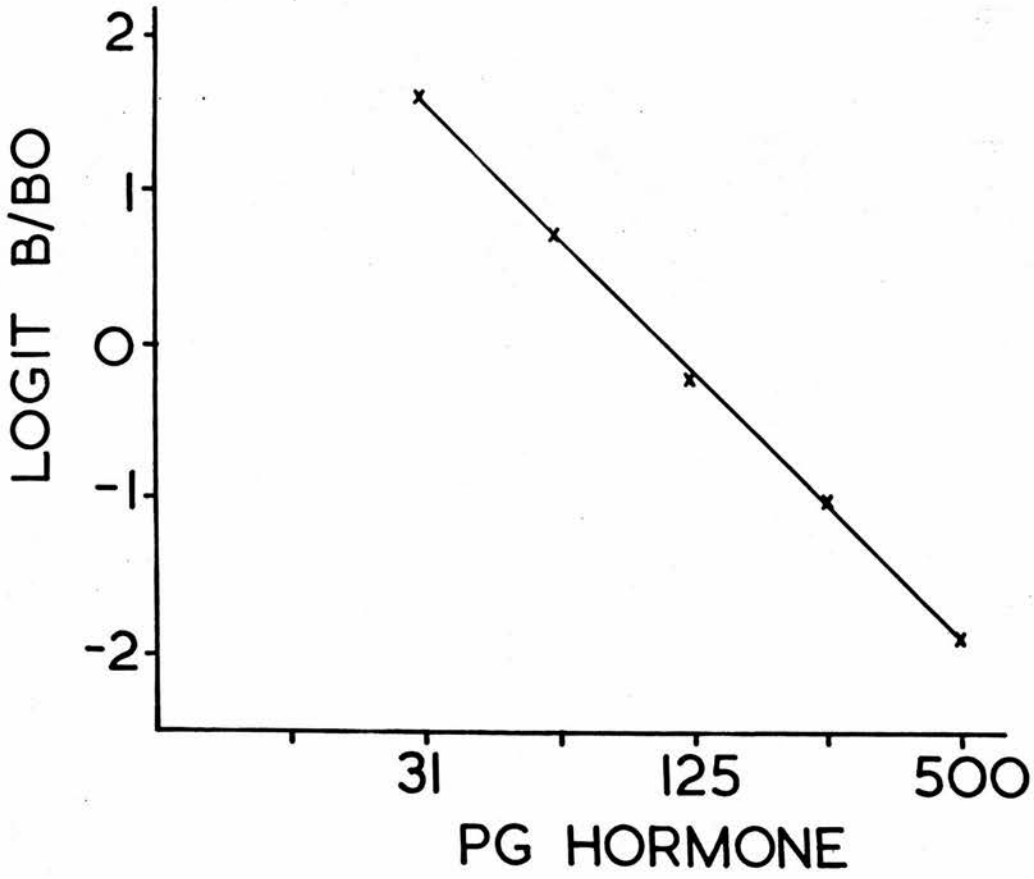


Figure 2.8

Standard curve for progesterone

Non radioactive testosterone (Sigma) was dissolved in ethanol to give a concentration of 1ug/ml, and stored at 4°C. 400ul were removed, evaporated to dryness, and the residue dissolved in 100mls of buffer, to give a concentration of 400pg/100ul. Suitable dilutions of this solution were then prepared to produce concentrations of 400, 200, 100, 50, 25 and 12.5pg/100ul buffer. These were used as standards.

Radioactive testosterone (1,2-^{3}H-testosterone) with a specific activity of 294 µCi/µg was obtained from New England Nuclear and stored at 4°C in ethanol. For use in the assay, an aliquot of this solution was dried down under nitrogen and redissolved in buffer to give a solution containing 6000 cpm of 1,2-^{3}H-testosterone per 100ul of buffer.

The antiserum was used at an initial dilution of 1:6000 in buffer.

Analytical grade hexane (BDH) and diethyl ether (BDH) were distilled separately (not more than 24 hours before use) and mixed in a ratio of hexane:ether (4:1). 1.0ml of this mixture was used to extract testosterone from plasma.

20ul 1,2-^{3}H-testosterone in ethanol (~1200 cpm) were added to the plasma (made up to 100ul with buffer) to test the recovery of testosterone.

The coefficient of variation for recovery between samples within an assay was approximately 6.7%. The range of recoveries was 69-82% over the first six assays.

Repeated assay of a pool of human plasma gave a mean value ±S.E.M. of 5.1 ±0.2 ng/ml, and inter-assay variation, expressed as the coefficient of variation was 12.7% (11 assays).

A typical standard curve for the testosterone assay is shown in Figure 2.9.

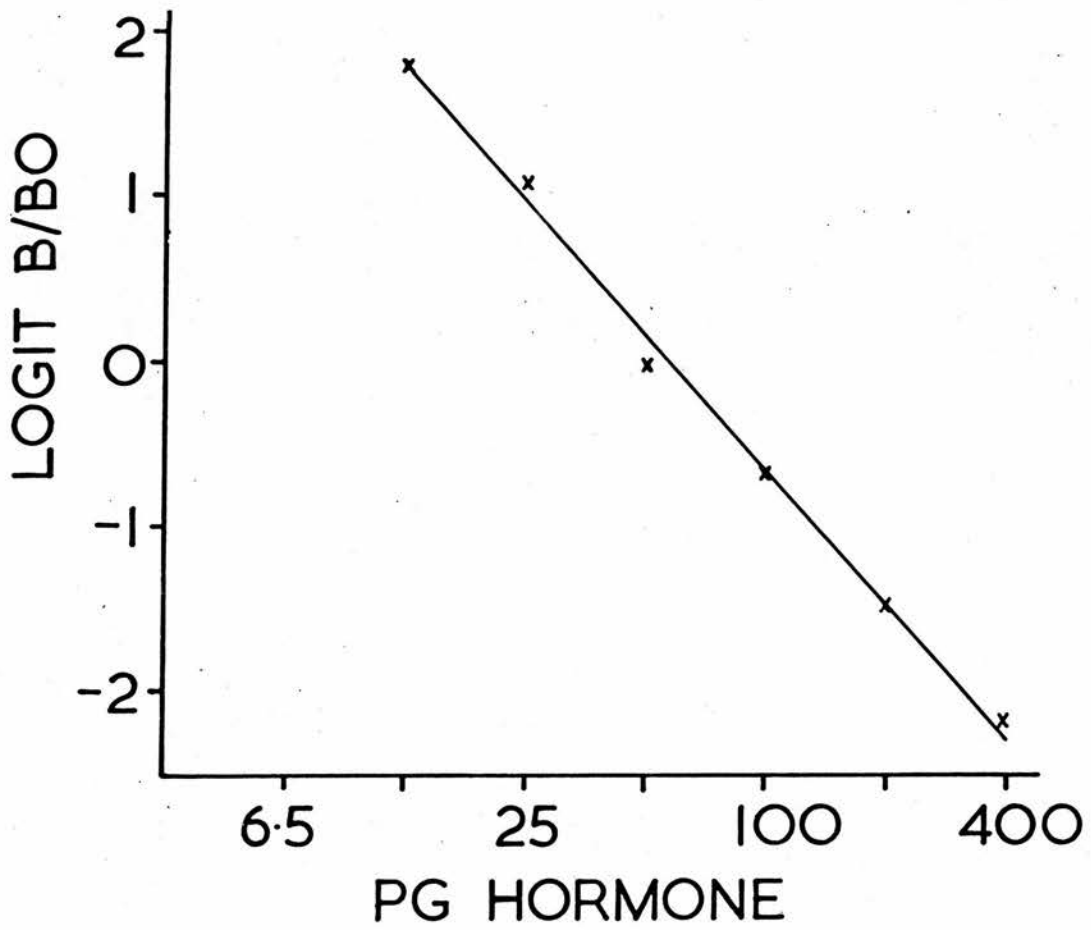


Figure 2.9

Standard curve for testosterone

2.14 Radioimmunoassay for Oestradiol-17β

Oestradiol-17β was measured by the method described by Baird, Swanston and Scaramuzzi (1976). The antiserum (OR - 422/7) was raised in sheep injected with oestradiol-6-keto-bovine serum albumen.

Cross reactions tested with other steroids included:- 6-keto-oestradiol, 100%; oestrone, 9.6%, oestradiol-17α, 2.8%; other steroids, <1%. The oestradiol-17β assay method is also similar to that described for progesterone apart from the following differences:

Non radioactive oestradiol-17β (Sigma) was stored in ethanol at 4°C at a concentration of 1 ug/ml. 512 ul were removed, evaporated to dryness, and the residue dissolved in 100 mls of buffer. Suitable dilutions of this solution were then prepared to give concentrations of 512, 256, 128, 64, 32, 16 and 8 pg/100 ul buffer. These were used as standards.

Radioactive oestradiol-17β (6,7-^{3}H-oestradiol-17β) with a specific activity of 179 μCi/μg was obtained from New England Nuclear and stored at 4°C in ethanol. In the assay, approximately 6000 cpm of 6,7-^{3}H-oestradiol-17β (i.e. 35-40 pg) per 100 ul buffer were added to each tube.

Aliquots of 50 ul plasma were made up to 100 ul with buffer. To test recovery of oestradiol-17β from plasma 20 ul of 6,7-^{3}H-oestradiol-17β in ethanol (~ 1200 cpm) were added, and extracted as for progesterone.

Oestradiol-17β was extracted from plasma with 1.0 ml of analytical grade diethyl ether (BDH) which had been washed with 50% (^{W}/v) ferrous sulphate (BDH) in 5% (^{W}/v) sulphuric acid (BDH) and distilled water, and redistilled within 24 hours of use.

The coefficient of variation for recovery between samples within an assay was approximately 6.1%. The range of recoveries was 76-87% over the first 6 assays.

Repeated assay of a pool of marmoset plasma gave a mean ($\pm$S.E.M.) value of 20.9 $\pm$ 0.9 ng/ml, with a coefficient of variation of 11.9% (8 assays).

A typical standard curve for the oestradiol-17β assay is shown in Figure 2.10.

Cross-reaction of oestradiol benzoate in the oestradiol-17β assay

20mg oestradiol benzoate (Sigma) were dissolved in absolute ethanol (10 ml). 20 ul of this solution were diluted to 50 ml in buffer to give a concentration of 100 ng/100 ul. Serial dilutions of this solution were prepared and used to construct a standard curve. A normal standard curve for oestradiol-17β was also included. 50% inhibition of binding was achieved with 76pg oestradiol-17β and 20 ng oestradiol benzoate. Cross reaction of oestradiol benzoate in the assay is therefore 0.38% (Fig. 2.11).

2.15 Measurement of anti LH-RH antibodies in plasma

2.15.a Iodination of LH-RH

Synthetic LH-RH was labelled with $Na^{125}I$ by a modification of the chloramine-T method of Greenwood, Hunter and Glover (1963). Iodinations were carried out with the help of Dr. H.M. Fraser.LH-RH (0.8 - 1.0 ug in 100 ul 0 01M PBS, pH 7.5 + 25 ul 0.5M PBS) were reacted with $\sim$ 1.0mCi $Na^{125}I$ and 10 ug chloramine-T (in 10 ul 0.01M PBS). The reaction was stopped after 30 seconds by adding 50 ug sodium metabisulphite in 100 ul 0.01M PBS. Potassium iodide (2mg) was then added in 200 ul of the same buffer. Labelled LH-RH was

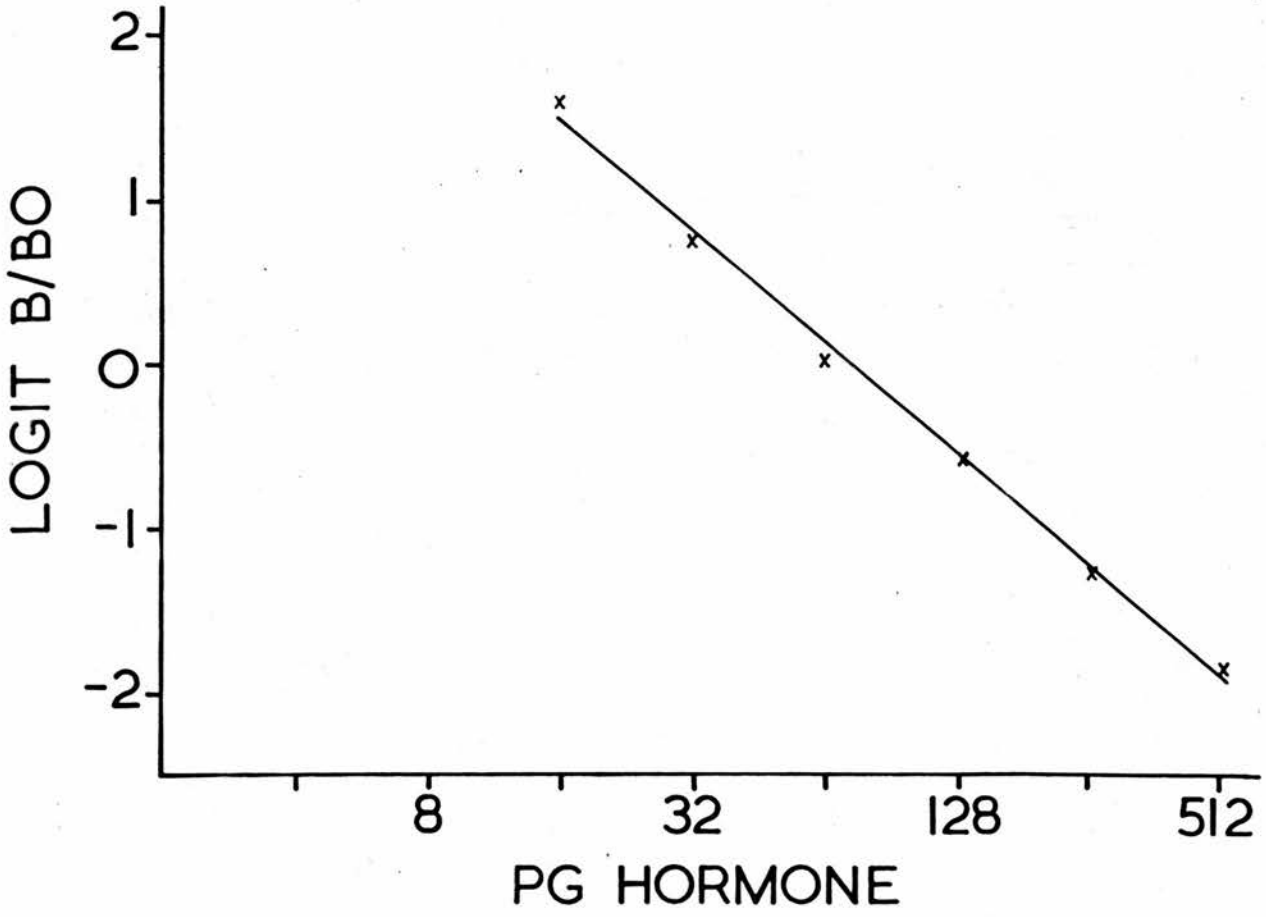


Figure 2.10

Standard curve for oestradiol-17β

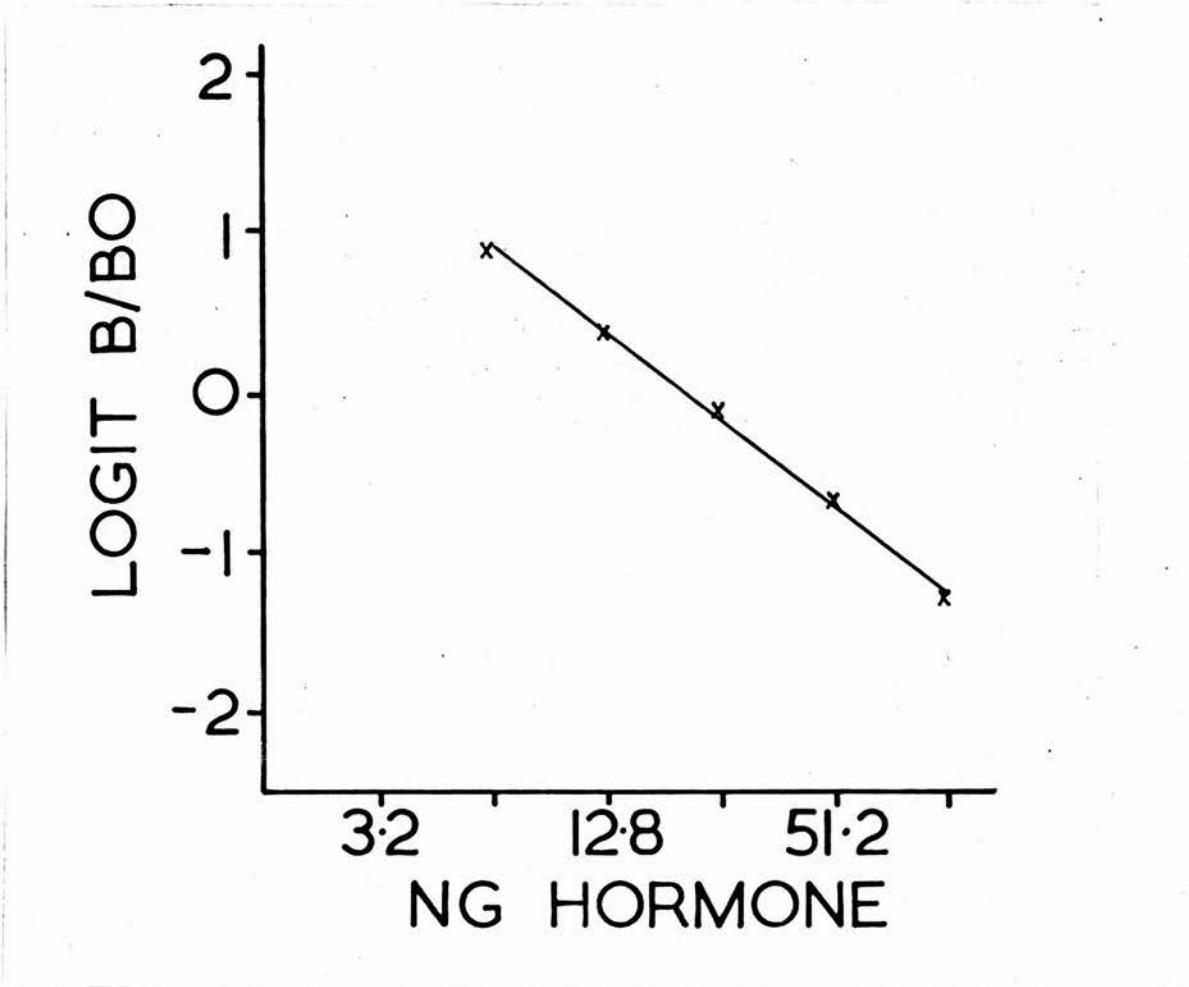


Figure 2.11

Standard curve for oestradiol benzoate

isolated from the reaction mixture by adsorption chromatography on a column (1 x 8 cm) of Whatman CF 11 grade cellulose. The reaction mixture was applied to the column which was then washed with 30 ml 0.01M PBS. The ^{125}I-labelled LH-RH was eluted from the column by passing through 15 ml of 0.4 M PBS containing 1% BSA, and collecting 1ml fractions. The most immunoreactive fractions were pooled and repurified immediately before use, as follows:

A 5 - 10 cm column of CF 11 cellulose was equilibrated with 0.01M PBS. ^{125}I-LH-RH was added to the column which was eluted with 20 ml of 0.01M PBS to remove free ^{125}I and damaged ^{125}I-LH-RH. 10 ml of 0.4M PBS containing 1% BSA were then passed through the column and the early fractions (1 ml) collected. In this way about 30% of the ^{125}I-LH-RH is recovered.

2.15.b Radioimmunoassay

Plasma was diluted tenfold with 0.01M PBS containing 1% BSA and stored at -20°C. For use in the assay this solution was diluted to 1 : 500 with 0.01M PBS containing 0.1% BSA. Samples were assayed in duplicate as follows:-

Each tube (10 x 75 mm plastic disposable) received 100ul of diluted plasma (i.e. antiserum), 100 ul of 0.01M PBS + 0.5% BSA, and 100 ul ^{125}I-LH-RH (approximately 10000 cpm (10 pg) in 0.01M PBS + 0.1% BSA). The contents of the tubes were mixed and incubated overnight at 4°C. Separation of antibody bound and free hormone was achieved by adding 1.5 mls of absolute ethanol (4°C), mixing thoroughly, and centrifuging at 4°C and 2500 rpm for 20 minutes. The supernatants were decanted, the tubes dried with tissue paper, and the antibody-bound ^{125}I-LH-RH in the precipitate was measured in an automatic gamma spectrometer.

Two tubes received 100 ul tracer (Total counts) and two tubes received 100 ul tracer, 100 ul PBS + 0.1% BSA and 100 ul PBS + 0.5% BSA (Non-specific binding). The mean non-specific binding value was subtracted from all plasma values. The amount of ^{125}I-labelled LH-RH bound by the plasma (expressed as a % of the total counts) was used as a measure of antibody titre.

CHAPTER THREE

INHIBITION OF LUTEINISING HORMONE RELEASING HORMONE ACTION

3.1 Introduction

Pituitary gonadotrophin release is stimulated by luteinising hormone-releasing hormone (LH-RH) isolated from hypothalamic extracts and characterised in 1971 as a decapeptide (Matsuo, et al, 1971). Administration of natural or synthetic LH-RH causes release of LH and a comparatively slight release of FSH in several species (Schally, Arimura and Kastin, 1973). One way in which the action of LH-RH may be studied is by its inhibition or elimination in vivo. One approach has been the production of lesions or islands in selected areas of the hypothalamus (Halász, 1969; Donovan, 1970) but recently, after the synthesis of LH-RH (Matsuo et al, 1971) more specific forms of inhibition by chemical means have become possible. In this way selective control of pituitary gonadotrophin secretion can be achieved either immunologically by the production of specific antibodies, or by the use of peptide analogues of LH-RH which antagonise the action of the endogenous hormone.

Selective neutralisation of LH-RH by specific antibodies can be achieved by active (long term) and passive (short term) immunisation. Active immunisation against LH-RH, which has been reported in rodents (Fraser, Gunn, Jeffcoate and Holland, 1974; Fraser, 1976a,b; Arimura Shino, de la Cruz, Rennels and Schally, 1976) and more recently in sheep (Clarke, Fraser and McNeilly, 1977), results in gonadal atrophy and inhibition of reproductive function. Antagonistic analogues of LH-RH competitively inhibit the action of LH-RH on the pituitary gland. In this way some of the more active peptides are able to suppress LH-RH induced LH release and block ovulation in rats (Corbin and Beattie, 1975; de la Cruz, Vilchez-Martinez, Arimura and Schally, 1976; Nishi, Coy, Coy, Arimura and Schally, 1976). In view of the possible

implications of specific LH-RH inhibition in terms of human fertility control, it would be useful to test these new methods in primates.

The present chapter is concerned with inhibition of LH-RH in the marmoset monkey. It deals firstly with active immunisation against LH-RH and outlines some of its effects on gonadal activity and pituitary function. In addition, a pilot study was performed to test the effectiveness of two of the more recently synthesised inactive analogues of LH-RH in inhibiting LH-RH induced LH release.

3.2 Active immunisation against LH-RH

3.2.a Procedure

Immunisation against LH-RH conjugated to BSA was performed in two stages: Group 1 (3 males, 3 females) was immunised in February, 1975; Group 2 (2 males, 2 females) was immunised 11 weeks later, in April 1975. Booster injections were given 10 weeks (group 1) and 11 and 46 weeks (group 2) after primary immunisation. Control animals (2 males, 2 females) were immunised against BSA.

Blood samples were taken and testicular and uterine dimensions were measured using calipers, according to the following schedule:-
LH-RH immunised animals: once weekly for 25 weeks (group 1) or 14 weeks (group 2), and then once every 5-10 weeks for the rest of the study (90 weeks).
BSA immunised animals: once every 2 weeks for 20 weeks.

Blood samples were assayed for antibody titres, progesterone, testosterone, and initially, LH. The presence of antibodies to LH-RH was assessed by measuring the percentage binding of approximately 10pg radioiodinated LH-RH by plasma at a dilution of 1:500 . The volumes of left testes were calculated from measurements of their

length and breadth using the formula $V = {}^1/6\pi B^2 L$, where V = volume (of an oblate spheroid), B = breadth and L = length.

Unilateral orchidectomy was performed after 53 weeks (group 1) and after 67 weeks (group 2). The size and appearance of ovaries were noted either during laparotomy after 20 weeks (group 1), or following unilateral ovariectomy after 53 weeks (group 1) and after 73 weeks (group 2). Testes and ovaries were removed from controls approximately 30 weeks after immunisation. Testes and ovaries were trimmed and fixed in Bouin's solution for histological examination. Sections were stained with haematoxylin-eosin. The diameters of 30 seminiferous tubules from each testis were measured using a calibrated micrometer eye-piece.

LH-RH immunised animals received rapid i.m. injections of synthetic LH-RH (4, 10, and 50 ug in 0.1ml saline) 37-40 weeks after immunisation. Controls received 4ug LH-RH only, after 20 weeks. All animals were bled immediately before, and 45 mins after the LH-RH injection, and plasma samples were assayed for LH.

Group 1 animals will subsequently be referred to as ♂ or ♀ 1, 2 and 3; group 2 animals as ♂ or ♀ 4 and 5, and control animals as ♂ or ♀ 6 and 7.

Animals were initially caged in male-female pairs according to their numbers, i.e. ♂ 1 with ♀ 1, ♂ 2 with ♀ 2, etc. Four animals were later separated and placed with non-immunised partners to test their fertility (♂ 1 and ♀ 1 68 weeks after immunisation; ♂ 2 and ♀ 2 after 61 weeks).

3.2.b Results

All animals immunised with LH-RH-BSA conjugate produced antibodies to LH-RH. In 6 animals (3 males, 3 females) antibody titres

were relatively high (47-57% binding), whereas in the other four, titres remained low (< 15% binding). Towards the end of the study problems were experienced in the preparation of suitably iodinated LH-RH, resulting in alteration of the characteristics of the assay for antibody titres. Consequently some of the results have not been included and the data on antibody titres are complete only for the first 65-75 weeks after primary immunisation.

No antibodies to LH-RH were produced by BSA immunised controls (< 1% binding) (Figs. 3.1 and 3.2). In the males (♂ 6, ♂ 7) there was no apparent inhibition of testosterone secretion and although testicular volume fluctuated slightly, there was no overall decrease. Both females (♀ 6, ♀ 7) were found to be pregnant 4 weeks after immunisation, and in each case pregnancies were continued to term after gestation periods of 20 and 21 weeks respectively.

In all three males which developed high antibody titres (♂1, ♂4, ♂5) there was suppression of testosterone secretion and marked testicular atrophy (Figs. 3.3 and 3.4). Antibodies were detected in the circulation 4-5 weeks after immunisation and levels continued to rise steadily until approximately week 20. Antibody titres rose following the first booster injection, although the second booster (♂4, ♂5) appeared to have little effect on the levels of antibody. As titres increased, testes became softer and smaller, eventually shrinking to less than 20% of their original volume. The development of high levels of antibody was also associated with a marked inhibition of testosterone secretion, and by 10-12 weeks after immunisation, plasma testosterone concentrations were no longer detectable (< 1ng/ml).

Figure 3.1

Testis volume, testosterone concentrations and antibody titres in two BSA immunised males (controls)

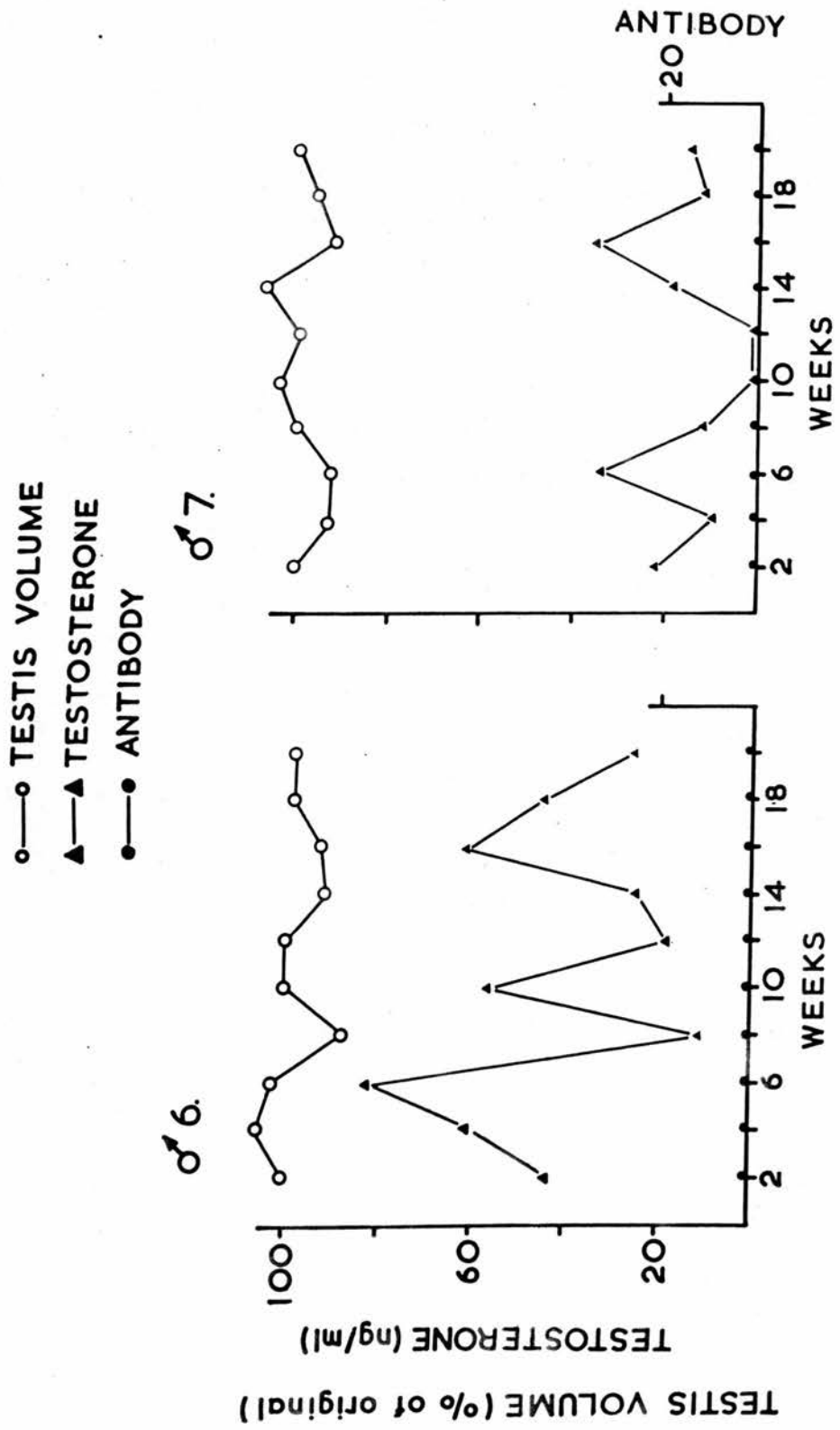
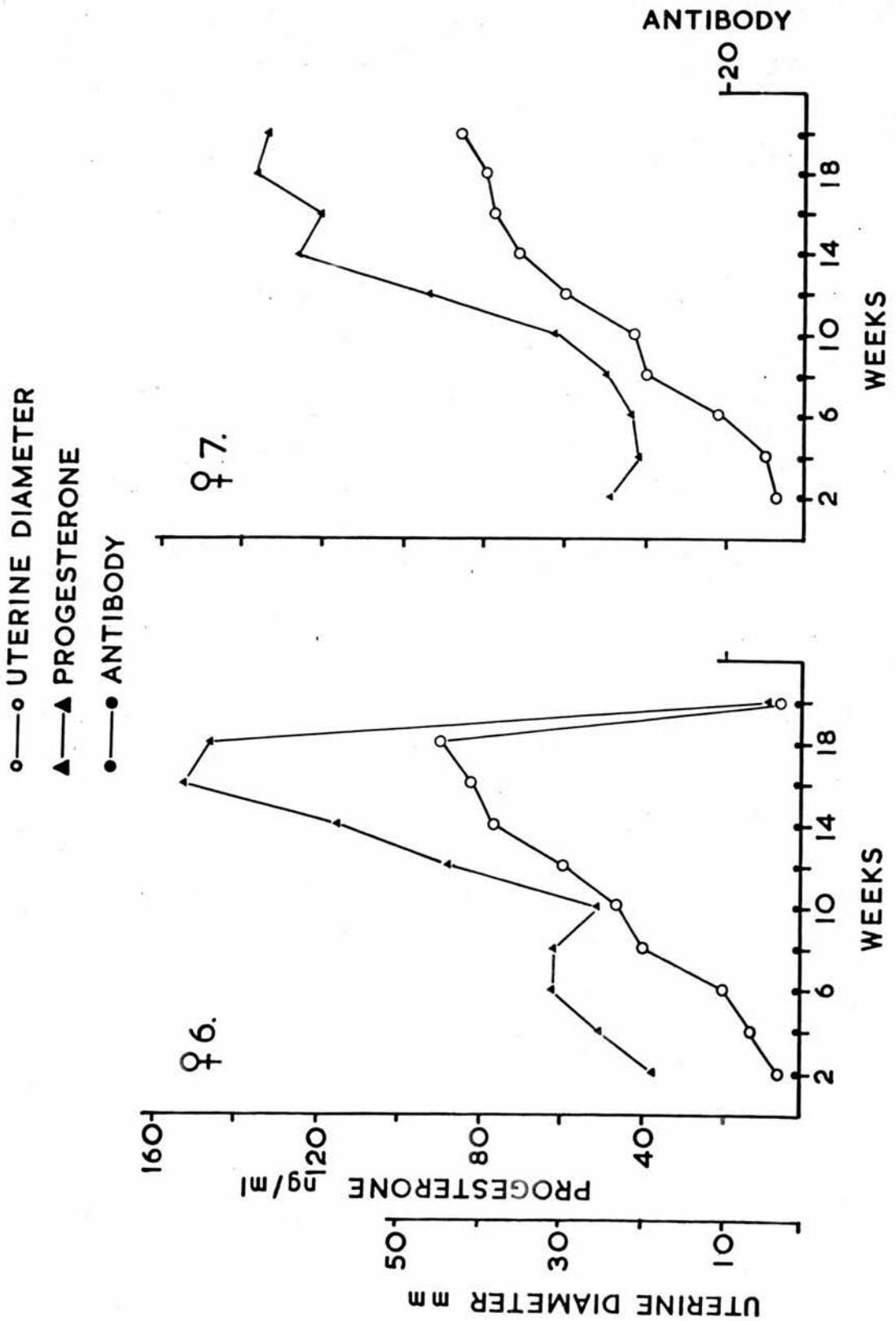


Figure 3.2

Uterine diameter, progesterone concentrations and antibody titres in two BSA immunised females (controls)



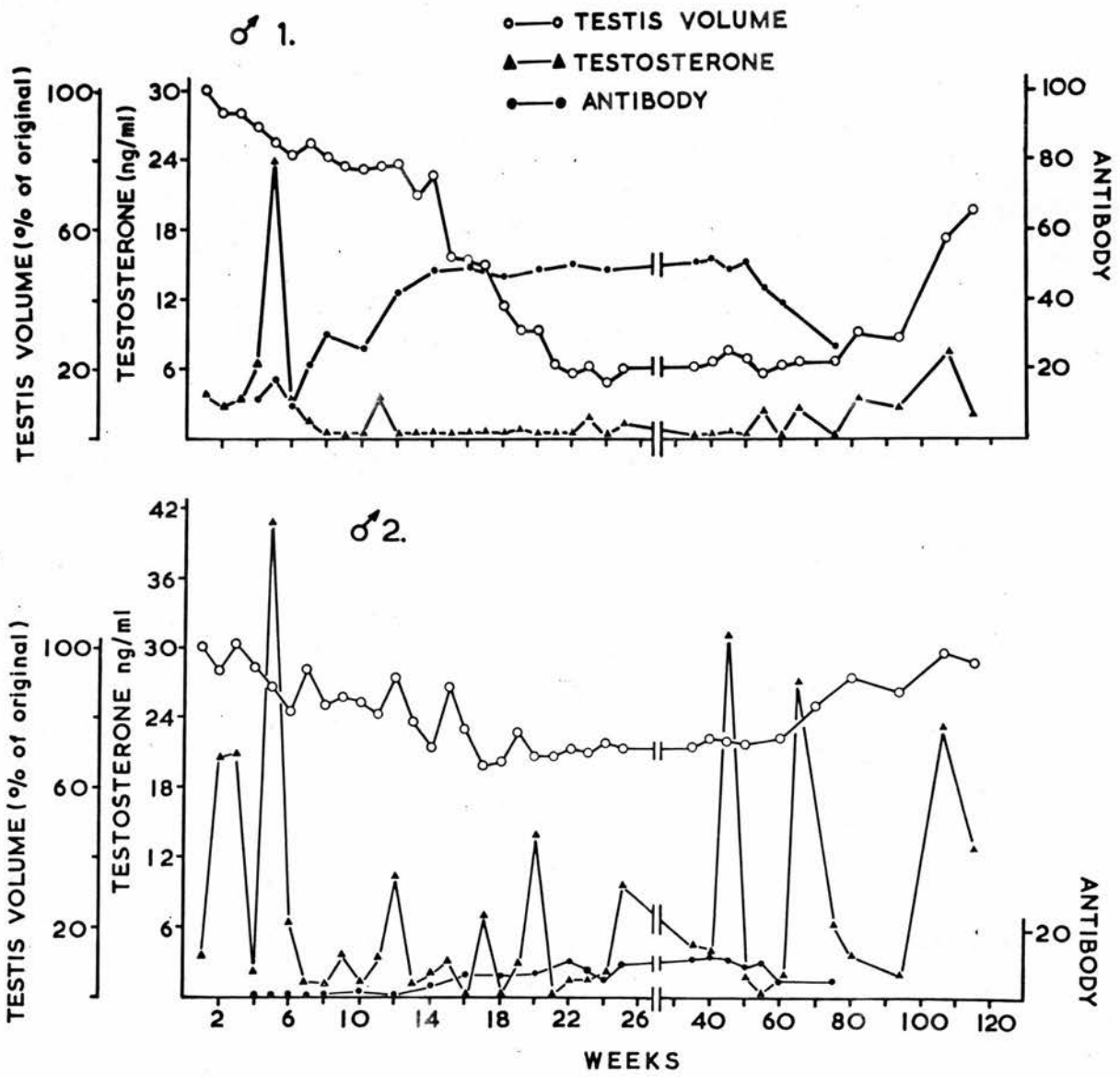


Figure 3.3

Testis volume, testosterone concentrations and antibody titres in two LH-RH immunised males

♂ 1, high antibody titre; ♂ 2, low antibody titre

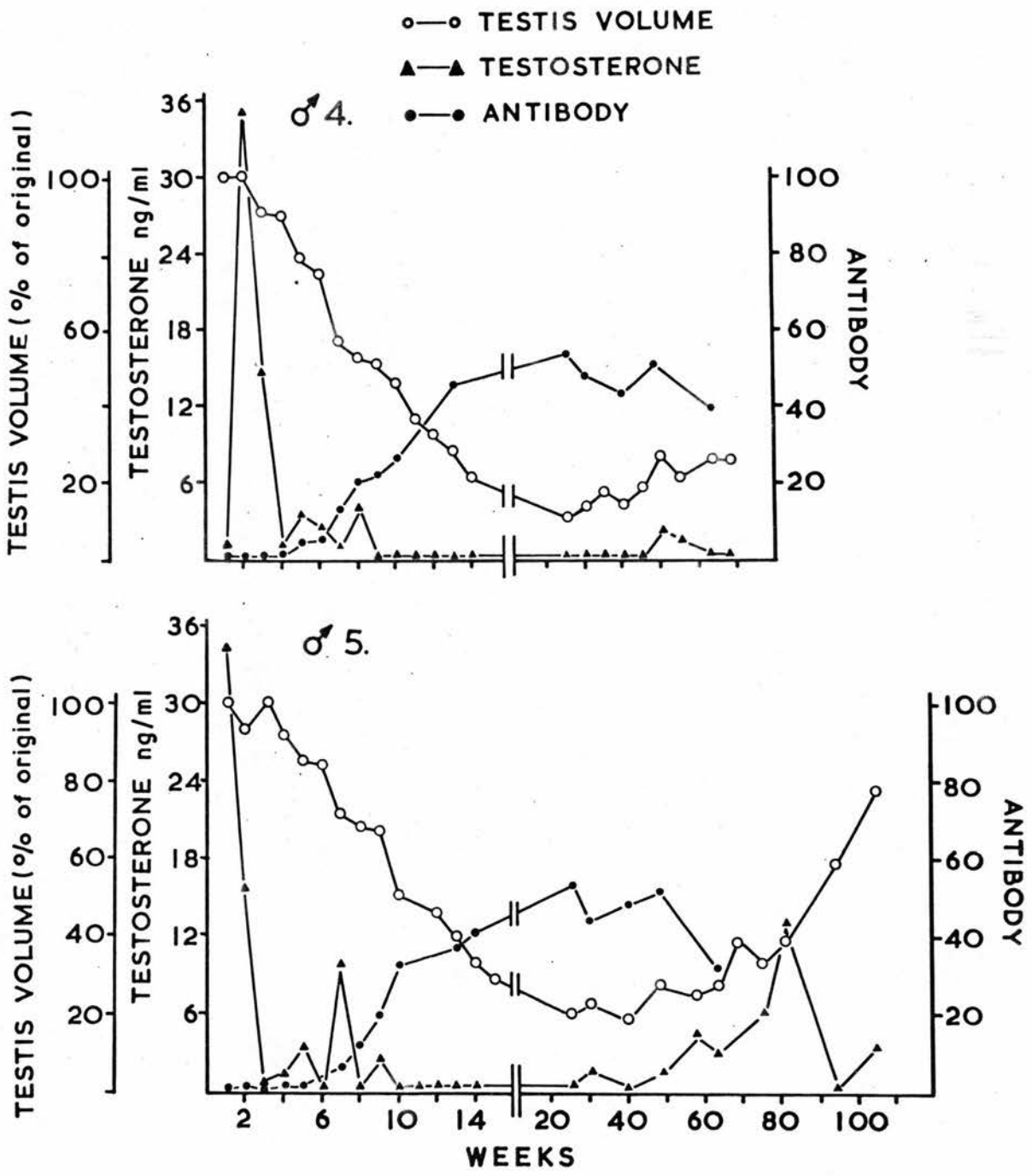


Figure 3.4

Testis volume, testosterone concentrations and antibody titres in two LH-RH immunised males

Both high antibody titres

The reduction in size and secretory activity of the testes was maintained throughout the period of high antibody titres. Levels of circulating antibodies began to fall approximately one year after primary immunisation in all three males, and an increase in testicular volume and testosterone production at this time could clearly be seen in ♂1 and ♂5. Although there was also a slight increase in testis volume in ♂4, testosterone secretion had not noticeably increased before the animal died 67 weeks after immunisation. Sixty eight weeks after immunisation ♂1 was placed with a non-immunised partner. This female was not observed to be pregnant until 48 weeks later, and 8 weeks after conception the pregnancy terminated in spontaneous abortion.

Low levels of circulating antibodies (♂2, ♂3) were relatively ineffective in suppressing testicular function (Fig. 3.4 & 3.5). Although a reduction in testis volume was observed in both animals (shrinkage was less than 40%), it was considerably less than in males with high antibody titres. The testes remained firm throughout the period of study, and began to grow again 60 (♂3) and 70 (♂2) weeks after immunisation. Sixty five weeks after immunisation ♂2 was placed with a non-immunised partner. Within 2 weeks this female became pregnant and gave birth to normal twins after a gestation period of approximately 20 weeks.

The effects of high levels of circulating LH-RH antibodies in females (♀ 1, ♀ 4, ♀ 5) are shown in Figures 3.6 and 3.7. One animal (♀ 1), which was 2 weeks pregnant at the time of immunisation, showed a much more rapid build up of antibody titres than the other two females. Antibody titres became relatively stable after 8 weeks and the booster produced no further increase. During the first 8 weeks the pregnancy continued normally, after which progesterone concentrations began to decrease and then fell rapidly

Figure 3.5

Testis volume, testosterone concentrations and antibody titres in an LH-RH immunised male

Low antibody titres

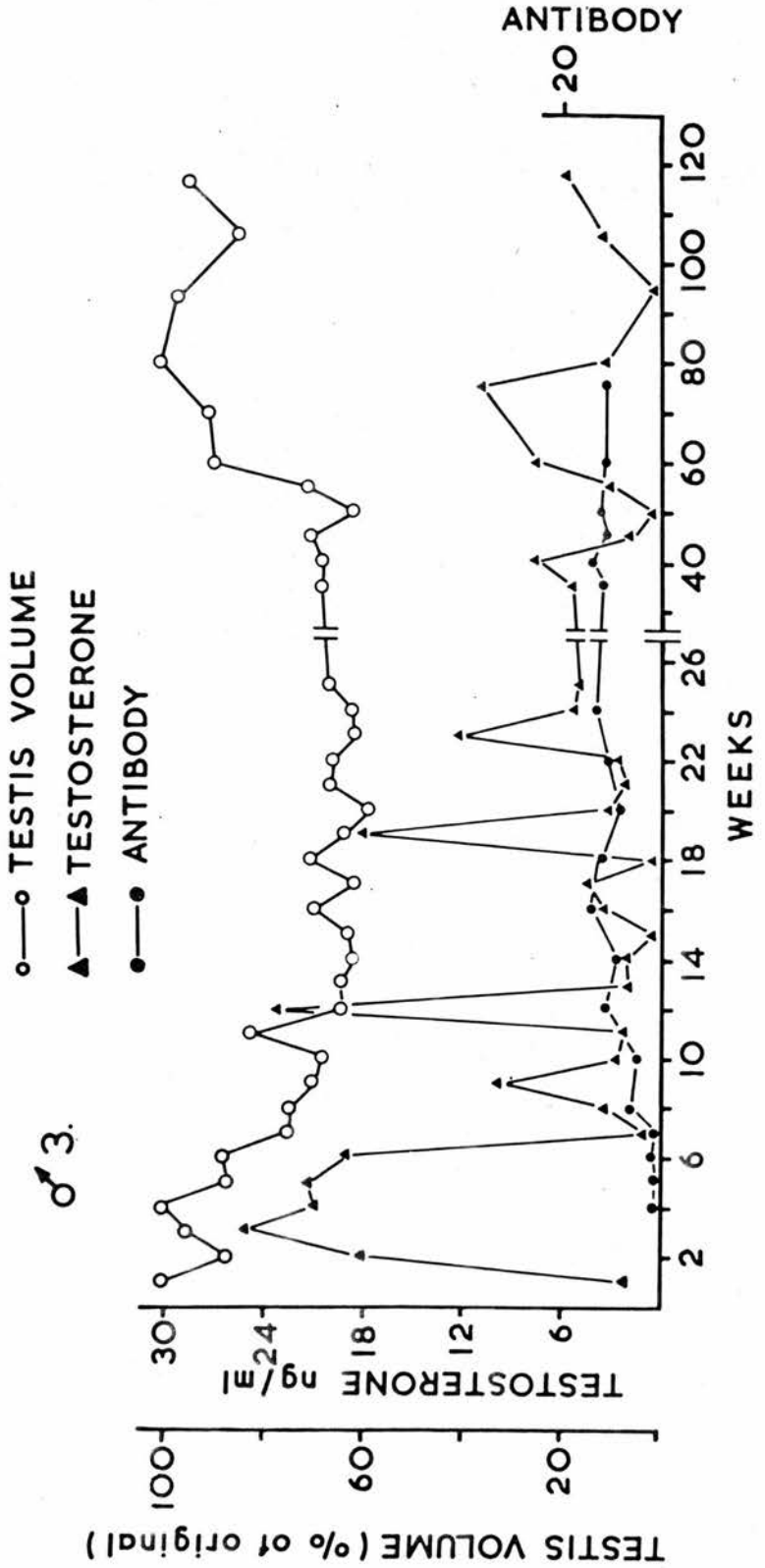
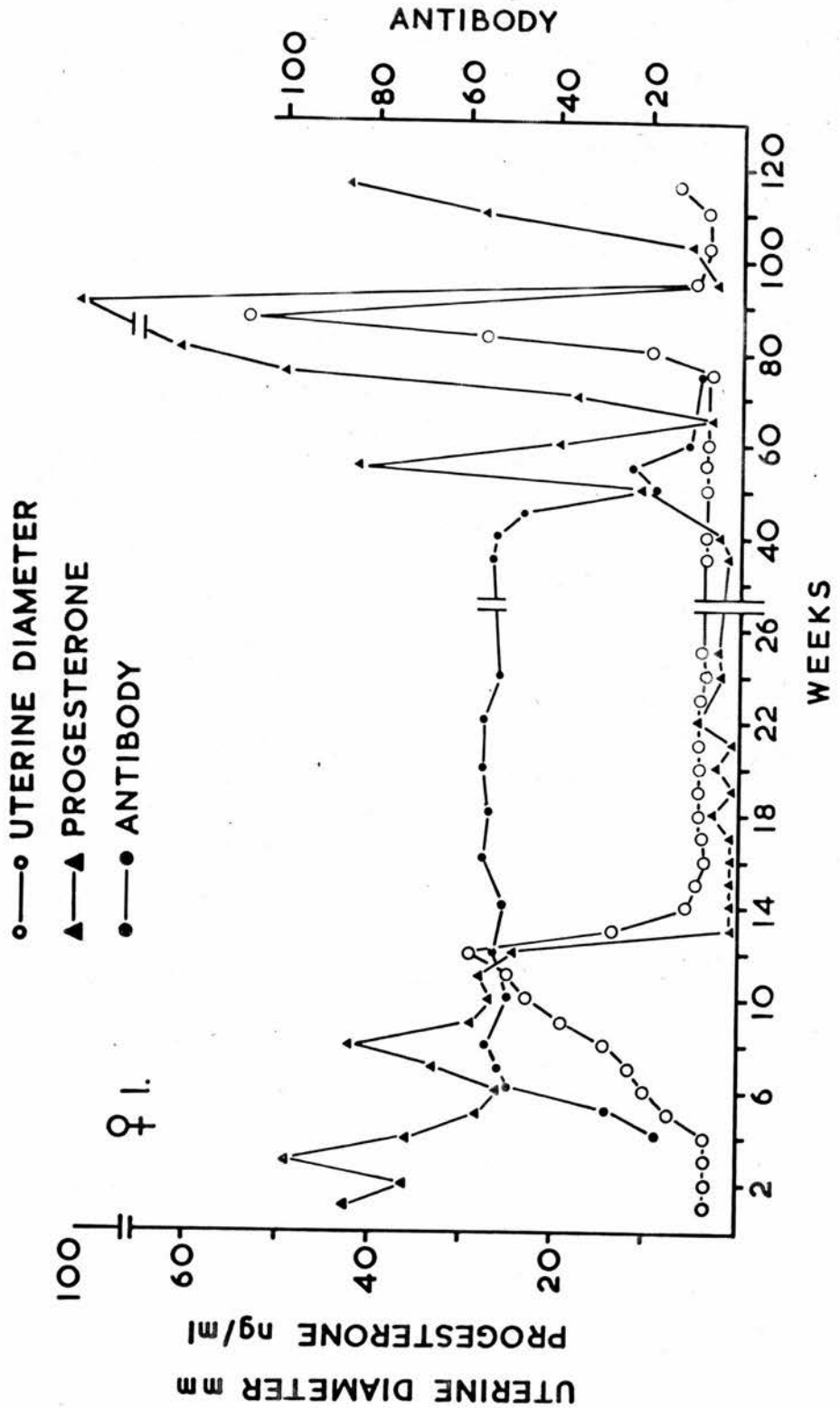


Figure 3.6

Uterine diameter, progesterone concentrations and antibody titres in an LH-RH immunised female

High antibody titres



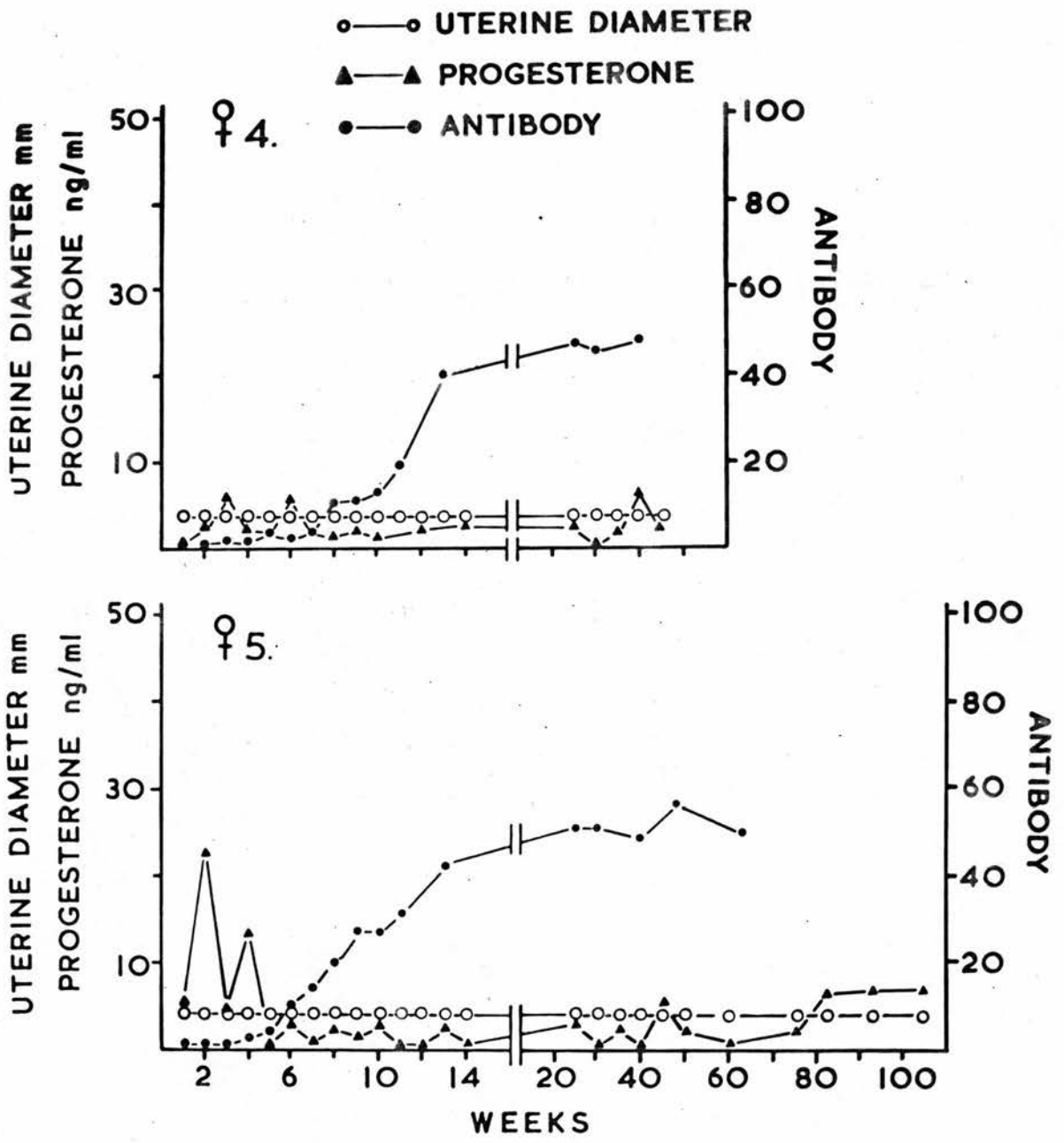


Figure 3.7

Uterine diameter, progesterone concentrations and antibody titres in two LH-RH immunised females

Both high antibody titres

at week 12, as the animal aborted. Progesterone concentrations remained low (< 5ng/ml) for up to 45 weeks after immunisation, showing that ovulation had ceased. Antibody titres then fell sharply and elevated progesterone levels indicated that ovarian cycles had recommenced. At this time (68 weeks after immunisation), ♀ 1 was placed with a normal male and within 7 weeks pregnancy was noted, thus confirming the return to normal ovarian function in this animal. The pregnancy continued to term and healthy twins were born. Eighteen weeks later this animal was again pregnant and gave birth to triplets.

In the other two females (♀ 4, ♀ 5), the initial gradual increase in antibody titres was accelerated by the first booster injection although there was no apparent further increase following the second booster. No signs of ovarian cyclicity were observed in ♀ 4 during the period of study, and high antibody titres were maintained until the animal died 48 weeks after immunisation. Progesterone levels in ♀ 5 show that ovulation had ceased by 6 weeks after immunisation, and no further evidence of ovarian cyclicity could be observed throughout the rest of the study.

Of the two females with low antibody titres (Fig. 3.8), ♀ 3 failed to show any evidence of ovulation between the time of immunisation and death 38 weeks later. The other female (♀ 2) was clearly experiencing ovulatory cycles during the first 12 and last 50 weeks of the study, although progesterone levels suggest that ovulation did not take place during the intervening period. Although there were signs of ovarian cyclicity soon after ♀ 2 was paired with a normal male (61 weeks after immunisation), conception did not occur until 40 weeks later, and the pregnancy was terminated in spontaneous abortion after 13 weeks.

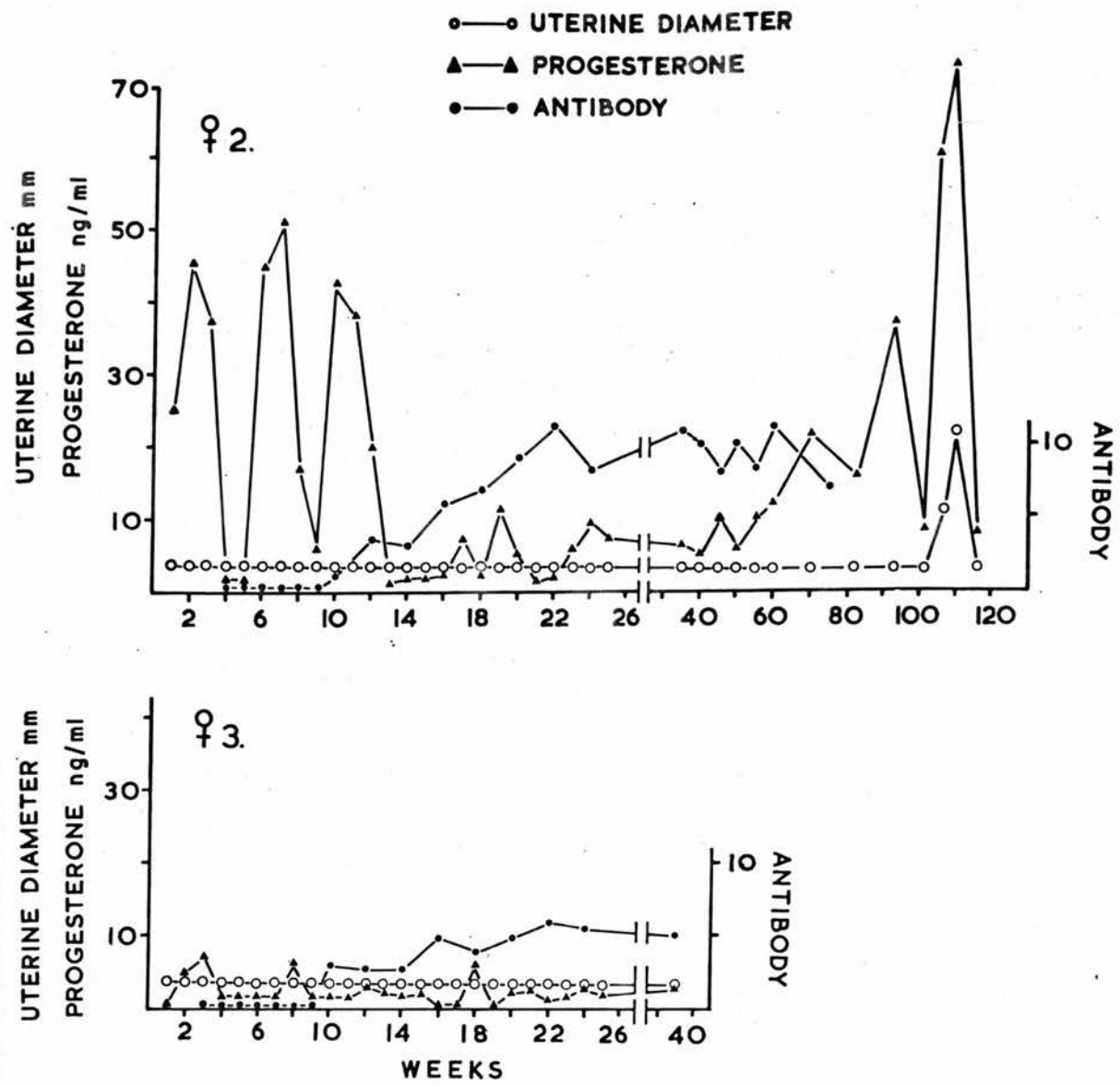


Figure 3.8

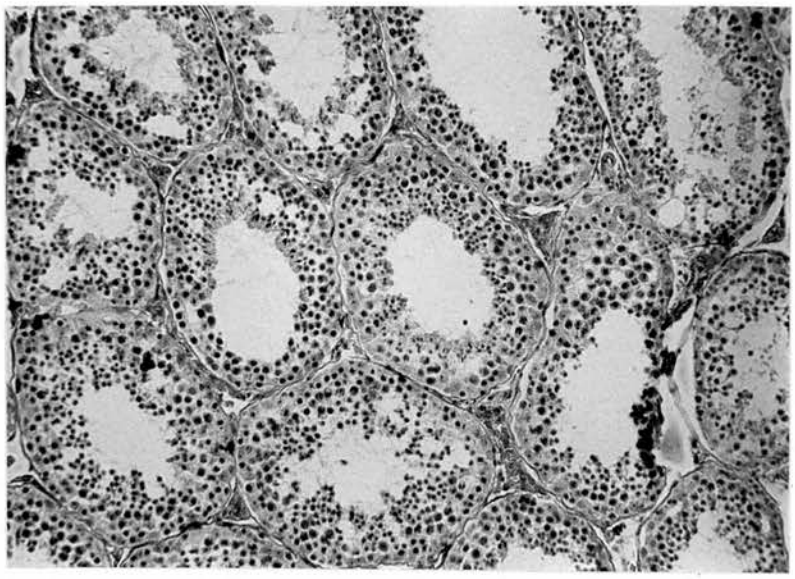
Uterine diameter, progesterone concentrations and antibody titres in two LH-RH immunised females

Both low antibody titres. N.B. Antibody scale has been enlarged to avoid excessive overlap.

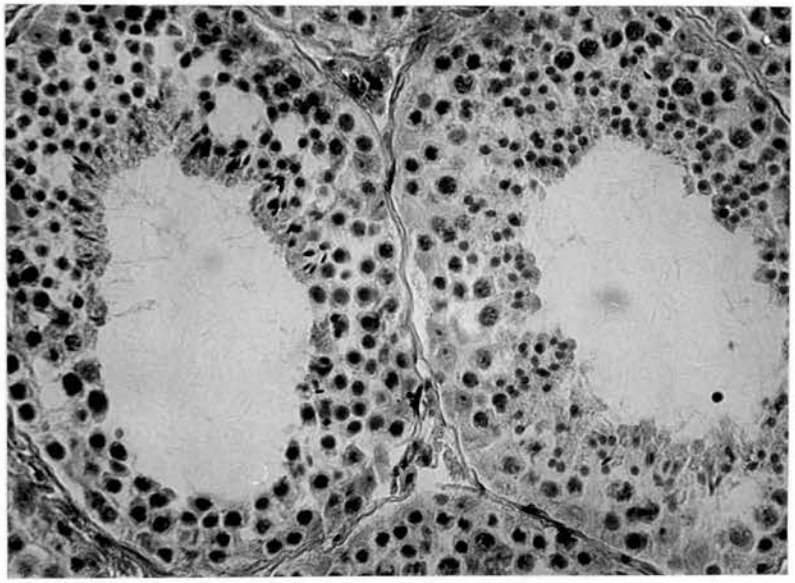
Basal levels of LH in marmosets are often below or only slightly above the limit of sensitivity of the LH assay. Consequently, it was not possible to detect any decrease in circulating LH concentrations after immunisation.

Histological examination of the testes revealed that spermatogenesis was proceeding normally in control animals (Fig. 3.9). In the three males with high levels of antibody there was a considerable reduction in seminiferous tubule diameter (Table 3.1), and spermatogenesis was severely impaired (Fig. 3.10). The tubules were lined with spermatogonia and contained spermatocytes up to the pachytene stage. There was however little spermatogenic development beyond this stage. There was also a reduction in the amount of interstitial tissue, and some of the cells contained condensed nuclei. Animals with low antibody titres showed intermediate effects. Although seminiferous tubules in these animals were also reduced in size, they were considerably larger than the tubules in animals with high antibody titres (Table 3.1), and there were no signs of spermatogenic arrest (Fig. 3.11).

Ovaries in females with high antibody titres were much reduced in size compared with ovaries in either control animals or animals with low antibody titres (Table 3.2). Compared with controls ovaries in animals with low titres were also reduced in size, but the difference was not significant. Ovarian histology revealed the presence of active luteal tissue in control animals (Fig. 3.12). The ovaries contained several primordial and pre-antral (single and multi-layered) follicles, and a relatively small number of antral follicles. The appearance of the luteal tissue suggests that the ovaries were removed during the early part of the luteal phase, and consequently there were no large



(a)



(b)

Figure 3.9

Light micrographs of the left testis from a BSA immunised marmoset (♂6, control)

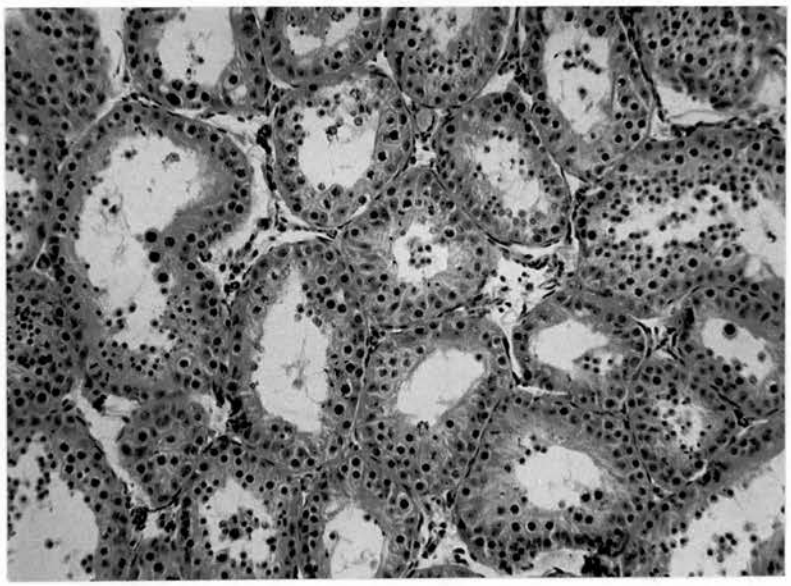
Haematoxylin and eosin stained. (a) x 102; (b) x 255.

	GROUP	ANIMAL	SEMINIFEROUS TUBULE DIAMETER (um) n=30 MEAN ± S.D.
1.	CONTROL	♂6	265.6 ± 25.5
		♂7	283.5 ± 34.5
2.	HIGH TITRES	♂1	139.8 ± 21.4
		♂4	129.9 ± 20.5
		♂5	136.6 ± 21.7
3.	LOW TITRES	♂2	243.6 ± 36.6
		♂3	230.0 ± 28.2

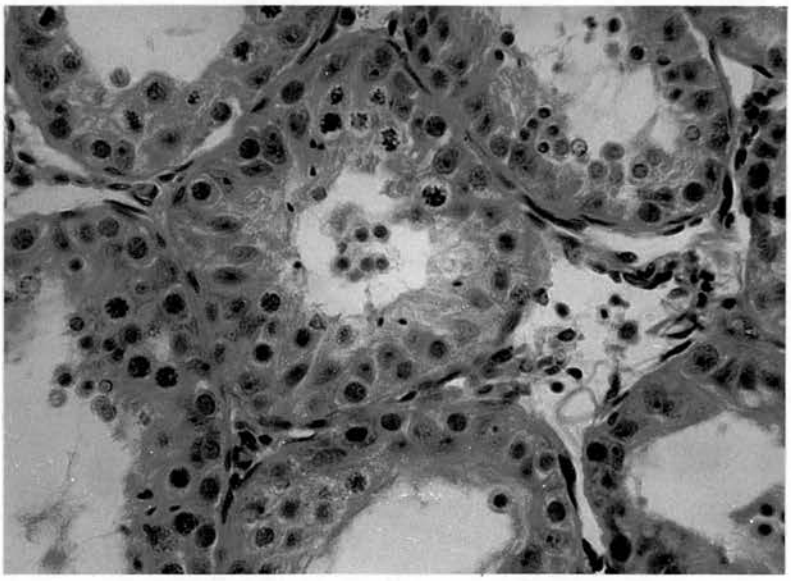
Table 3.1

Seminiferous tubule measurements in control and immunised male marmosets

For all animals $p < 0.001$ compared with the mean control values (students t-test)



(a)

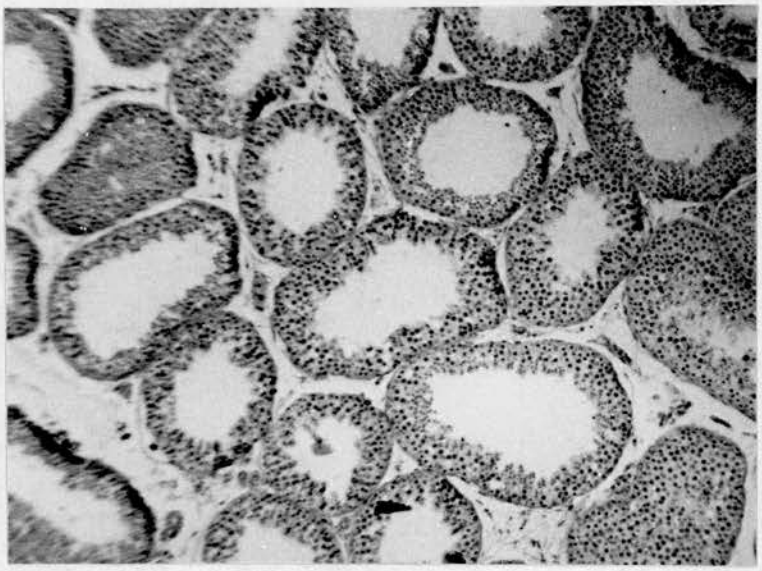


(b)

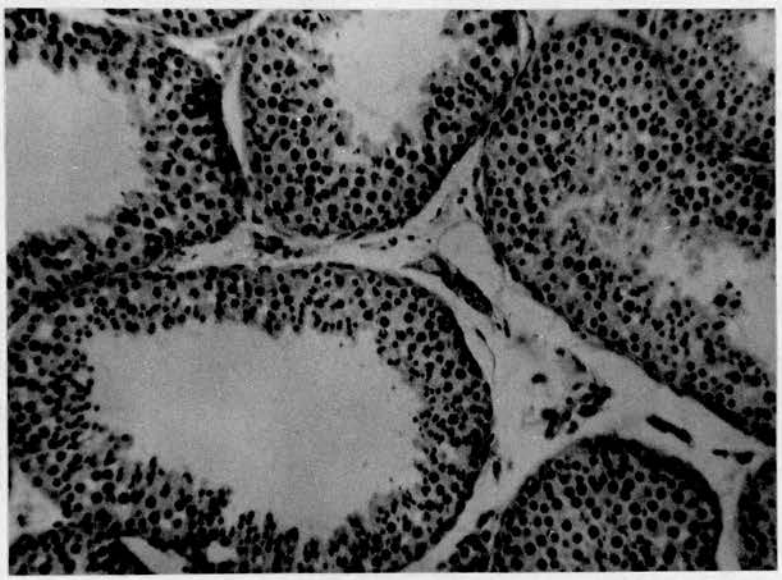
Figure 3.10

Light micrographs of the left testis from an LH-RH immunised marmoset (♂1, high antibody titres)

Haematoxylin and eosin stained. (a) x 138; (b) x 295.



(a)



(b)

Figure 3.11

Light micrographs of the left testis from an LH-RH immunised marmoset (♂2, low antibody titres)

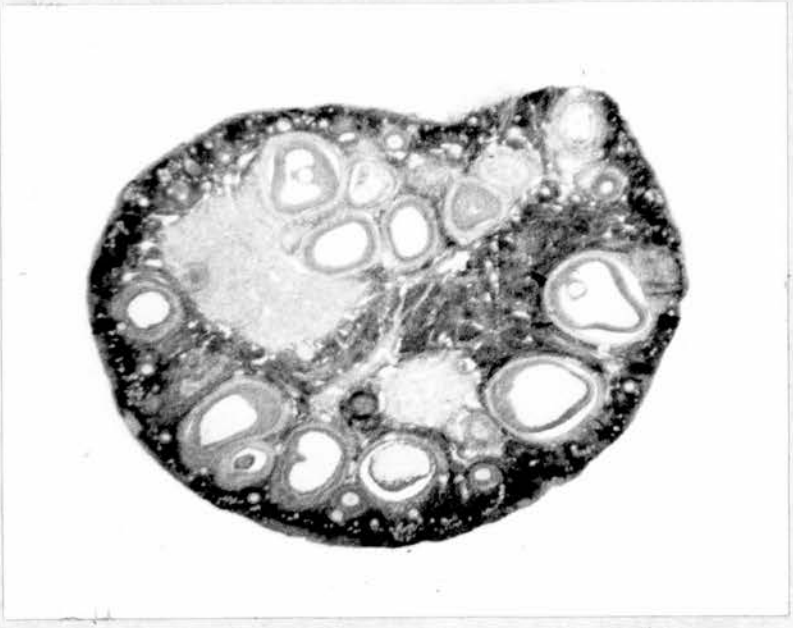
Haematoxylin and eosin stained. (a) x 110; (b) x 250.

GROUP	ANIMAL	LEFT OVARY		RIGHT OVARY	
		Dimensions (mm)	Length x breadth (mm^2)	Dimensions (mm)	Length x breadth (mm^2)
1. CONTROL	♀ 6	7.6 x 6.5	49.4	9.0 x 7.0	63.0
	♀ 7	8.0 x 6.2	49.6	8.8 x 6.8	59.8
	Mean ± S.D.		49.5 ± 0.1		61.4 ± 2.2
2. HIGH ANTIBODY TITRES	♀ 1	4.1 x 2.6	10.7	5.9 x 2.8	12.4
	♀ 4	5.5 x 2.5	13.8	5.4 x 2.5	13.5
	♀ 5	5.2 x 2.7	14.0	4.1 x 3.6	14.8
	Mean ± S.D.		12.8 ± 1.9		13.6 ± 1.2
3. LOW ANTI-BODY TITRES	♀ 2	4.5 x 4.0	18.0	6.0 x 5.3	31.8
	♀ 3	6.5 x 5.6	36.4	7.2 x 6.3	45.4
	Mean ± S.D.		27.2 ± 13.0		38.6 ± 9.6
1 vs 2			**		**
1 vs 3			-		-
2 vs 3			-		*

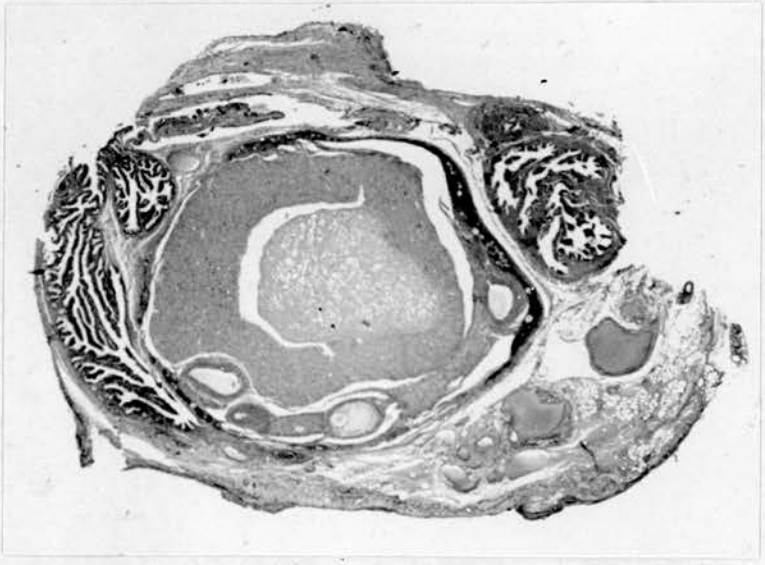
- not significant

** $p<0.01$ (students t-test)

Table 3.2: Ovarian size in control and immunised marmosets



(a)



(b)

Figure 3.12

Light micrographs of the left ovary from two BSA immunised marmosets (controls)

Haematoxylin and eosin stained. (a) ♀6 x 11.6; (b) ♀7 x 10.8

graafian or pre-ovulatory follicles. In contrast to the controls, none of the ovaries from LH-RH antibody producers contained luteal tissue. There were however many follicles present in the ovaries of all LH-RH immunised animals, although the extent of their development appeared to be related to the antibody titre. All stages of follicular development were seen in ovaries from animals with low antibody titres. Figure 3.13 shows two well developed antral follicles although they are not as large as a normal pre-ovulatory graafian follicle. There was also a slight "crowding together" of the follicles (compared with the controls) suggesting that there may have been a reduction in the amount of interstitial tissue. High levels of antibody had a more drastic effect on the ovaries, and only the early stages of follicular maturation could be observed. The ovary shown in Figure 3.14 contains many small, poorly developed follicles, the largest of which is at the pre-antral multi-layered stage. There were no antral follicles, and the amount of interstitial tissue was greatly reduced.

The effect of immunisation on the response of the pituitary to exogenous LH-RH is shown in Table 3.3. Animals with high antibody titres showed a much reduced response at all doses compared with animals with low antibody titres and with controls. A dose response relationship was demonstrated by high titre animals, whereas animals with low titres showed a similar response to all three doses of LH-RH. However, even in animals with high antibody titres that had been immunised for 40 weeks, the pituitary gonadotrophs still showed some response to the releasing hormone.

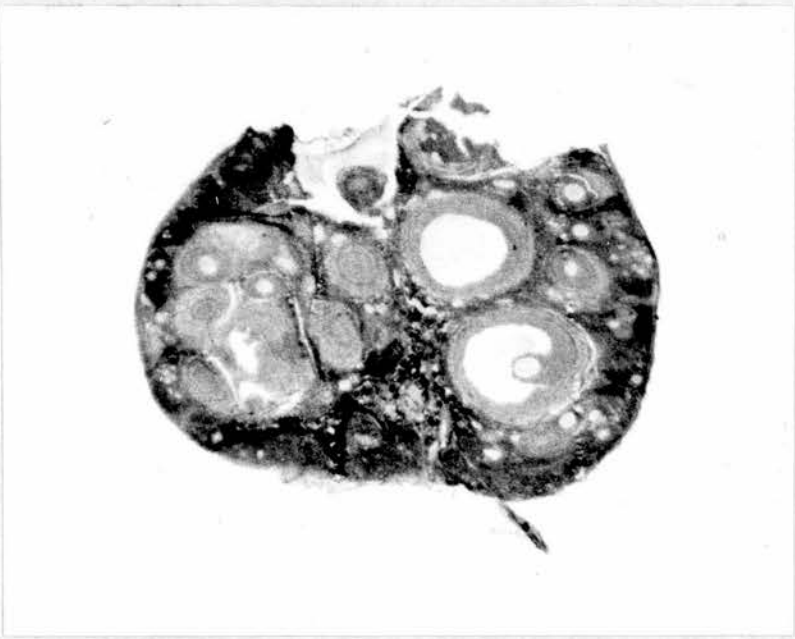
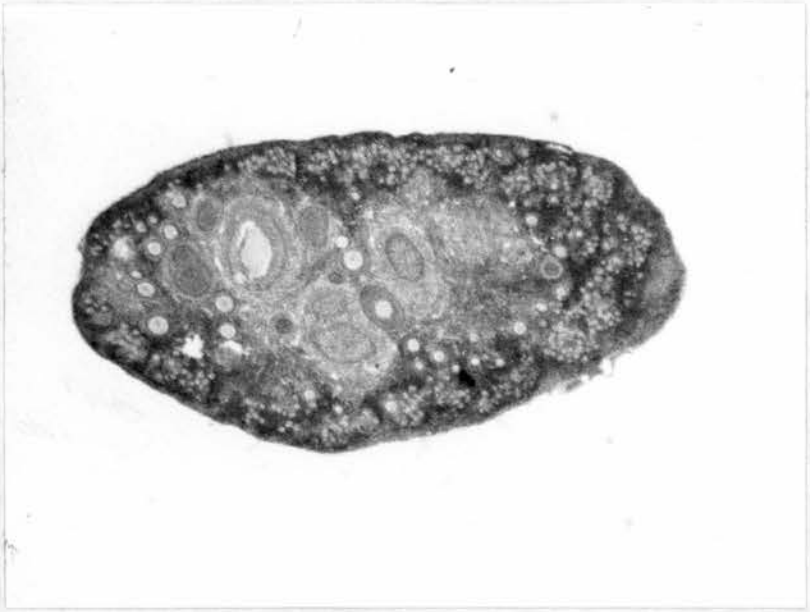


Figure 3.13

Light micrograph of the left ovary from an LH-RH immunised marmoset ($\overset{+}{o}$ 2, low antibody titres)

Haematoxylin and eosin stained, x 20.



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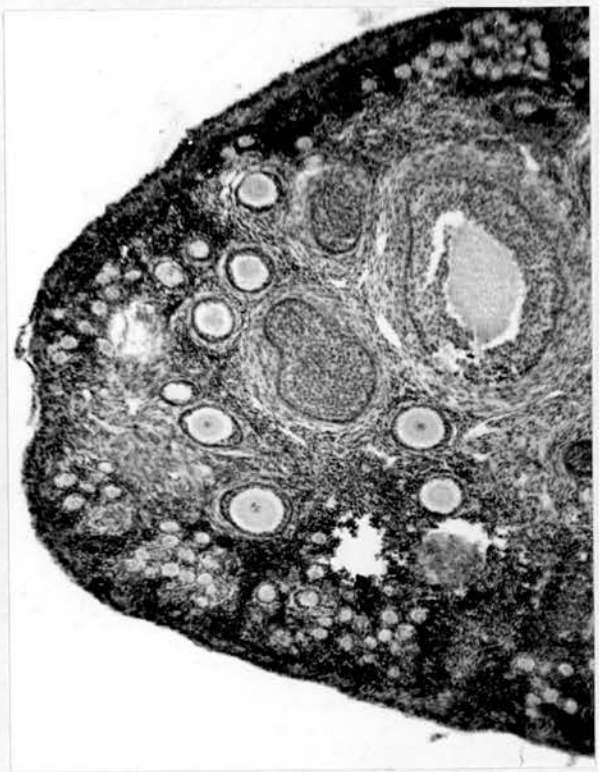

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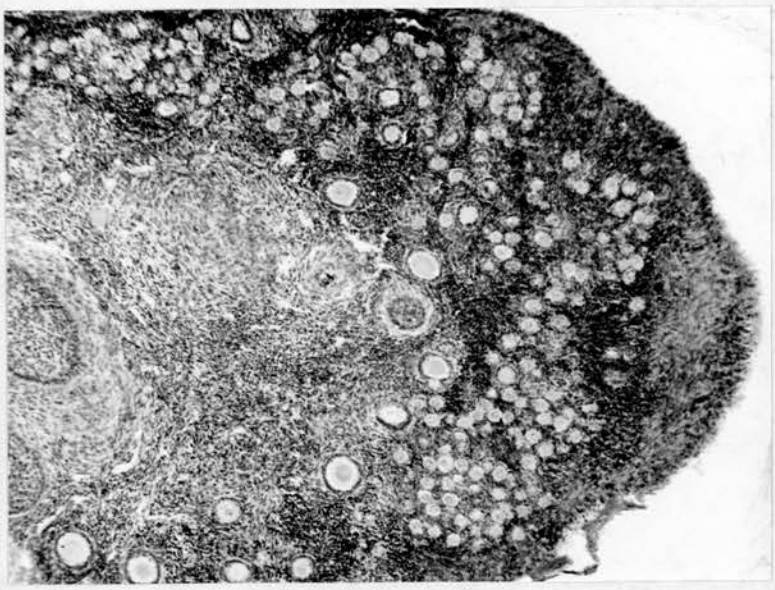


(c)

Figure 3.14

Light micrographs of the left ovary from an LH-RH immunised marmoset (♀ 5, high antibody titres)

Haematoxylin and eosin stained. (a) x 21; (b) and (c) x 50.

GROUP	ANIMAL	MALE			ANIMAL	FEMALE		
		4ug	10ug	50ug		4ug	10ug	50ug
HIGH TITRES	♂ 1	0^{+}	14^{*}	26	♀ 1	7^{*}	22^{*}	28
	♂ 4	0^{+}	6^{*}	23^{*}	♀ 5	0^{+}	4^{*}	28^{*}
	♂ 5	6^{*}	14	19^{*}				
LOW TITRES	♂ 2	79	63^{*}	67	♀ 2	72	68^{*}	63
	♂ 3	53	63	62^{*}				
CONTROL	♂ 6	81	-	-	♀ 6	87^{*}	-	-
	♂ 7	88	-	-	♀ 7	76	-	-

Table 3.3

Response to LH-RH in control and immunised marmosets

Figures represent the increase in LH above pre-injection levels (i.e. ΔLH)

* Pre-injection levels were undetectable (< 20ng/ml) and to calculate ΔLH, a value of 20ng/ml was substituted. A slight underestimation of ΔLH values has therefore resulted.

$^{+}$ Both pre- and post-injection levels were undetectable.

3.3 Inhibition of LH secretion with inactive analogues of LH-RH

3.3.a Procedure

The LH-RH analogues tested in this study were D Phe^2 Phe^3 D Phe^6 LH-RH (peptide A) and D Phe^2 D Trp^3 D Phe^6 LH-RH (peptide B).

For each analogue eight groups of intact male marmosets (n=3 per group) received the following treatment:-

Group 1. 0.1ml diluent only (20% propylene glycol in saline).

Group 2. Diluent and 0.5ug LH-RH in 0.1ml saline.

Groups 3-7. Analogue (1.0mg in 0.1ml diluent) and LH-RH, 0, 30, 60, 120 and 240 min later.

Group 8. Analogue only (1.0mg in diluent).

Analogue and diluent were given as subcutaneous injections, and LH-RH was injected intramuscularly. Blood samples were taken as follows:-

Group 1. Immediately before, and 30 min after the injection of diluent.

Groups 2 - 7. Immediately before the administration of diluent or analogue, and again 30 min after the LH-RH injection.

Group 8. Immediately before and 30, 60, 120 and 240 mins after the injection of analogue.

Plasma LH concentrations were measured in all samples.

3.3.b Results

The effects of LH-RH analogues on LH secretion in response to the administration of exogenous LH-RH are shown in Tables 3.4 and 3.5. Mean ($\pm$ S.E.M.) increases in LH concentrations above pre-injection levels are given in Figures 3.15 and 3.16. In the absence of analogue 0.5ug LH-RH induced a marked increase in plasma LH concentrations

ANIMAL	TREATMENT	LH (ng/ml)		Δ LH	LH (mean ± S.E.M.)
		0 min.	30		
1		43	40	-3	
2	DILUENT	20	20	0	-2.3 ± 1.2
3		68	64	-4	
4		20	126	106	
5	DIL. + LH-RH	31	114	83	95.7 ± 3.8
6		50	146	96	
7		68	152	84	
8	ANALOGUE + LH-RH	46	120	74	73.3 ± 6.4
9	0 MIN.	20	82	62	
10		21	82	61	
11	ANALOGUE + LH-RH	20	46	26	44.0 ± 10.1
12	30 MIN.	21	66	45	
13		66	98	32	
14	ANALOGUE + LH-RH	52	90	38	27.3 ± 7.9
15	60 MIN.	20	32	12	
16		20	66	46	
17	ANALOGUE + LH-RH	64	94	30	39.3 ± 4.8
18	120 MIN.	20	62	42	
19		52	96	44	
20	ANALOGUE + LH-RH	20	66	46	45.3 ± 0.7
21	240 MIN.	20	66	46	
		0	30 60 120 240		
22		66	66 64 52 52		
23	ANALOGUE + DIL.	47	48 41 29 32		
24		<20	<20 <20 <20 <20		
	MEAN	44.4	45.3 42.0 33.7 34.7		

Table 3.4

The effects of D Phe^2 Phe^3 D Phe^6 LH-RH on tonic LH secretion and LH-RH induced LH release in normal males

ANIMAL	TREATMENT	LH (ng/ml)		ΔLH	LH(mean ± S.E.M.)
		0 min.	30		
1	DILUENT	43	40	-3	-2.3 ± 1.2
2		20	20	0	
3		68	64	-4	
4	DIL. + LH-RH	47	152	105	105.3 ± 0.9
5		61	165	104	
6		62	169	107	
7	ANALOGUE + LH-RH 0 MIN.	47	117	70	67.0 ± 4.9
8		29	102	73	
9		24	81	57	
10	ANALOGUE + LH-RH 30 MIN.	20	34	14	22.7 ± 6.3
11		20	39	19	
12		30	65	35	
13	ANALOGUE + LH-RH 60 MIN.	37	95	58	46.0 ± 7.9
14		36	85	49	
15		22	53	31	
16	ANALOGUE + LH-RH 120 MIN.	20	57	37	39.0 ± 5.0
17		51	100	49	
18		20	52	32	
19	ANALOGUE + LH-RH 240 MIN.	40	131	91	93.8 ± 6.5
20		20	104	84	
21		20	126	106	

		0	30	60	120	240
22	ANALOGUE + DIL.	37	31	26	< 20	33
23		26	< 20	< 20	< 20	< 20
24		21	< 20	< 20	< 20	< 20
	MEAN	28.3	23.8	22	< 20	24.3

Table 3.5

The effects of D Phe^2 D Trp^3 D Phe^6 LH-RH on tonic LH secretion and LH-RH induced LH release in normal males

Figure 3.15

Time course in male marmosets of blockade of LH release in response to LH-RH by D Phe^2 Phe^3 D Phe^6 LH-RH (1.0mg per animal)

Analogue was injected at time 0. Values are mean ± S.E.M.

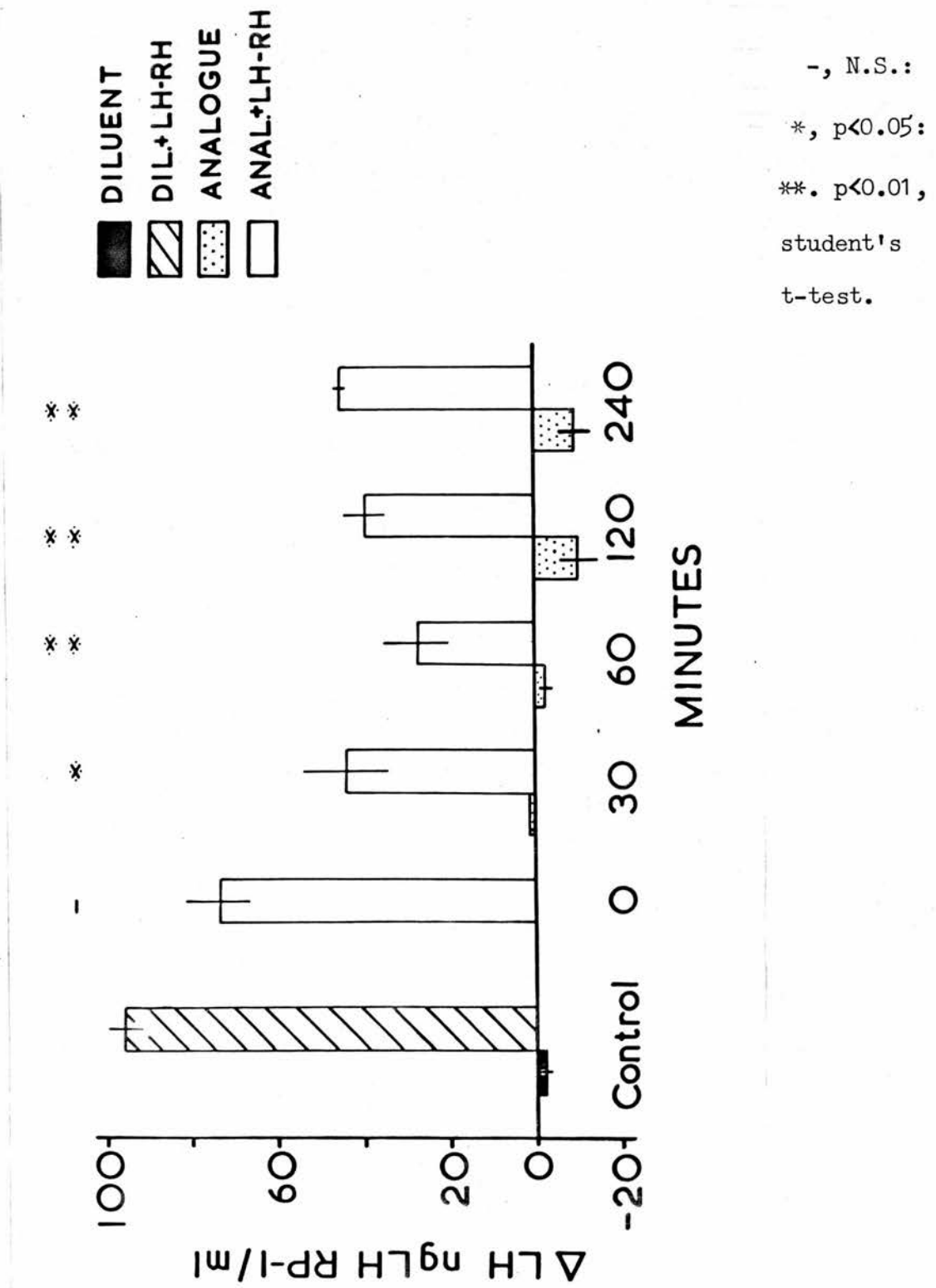
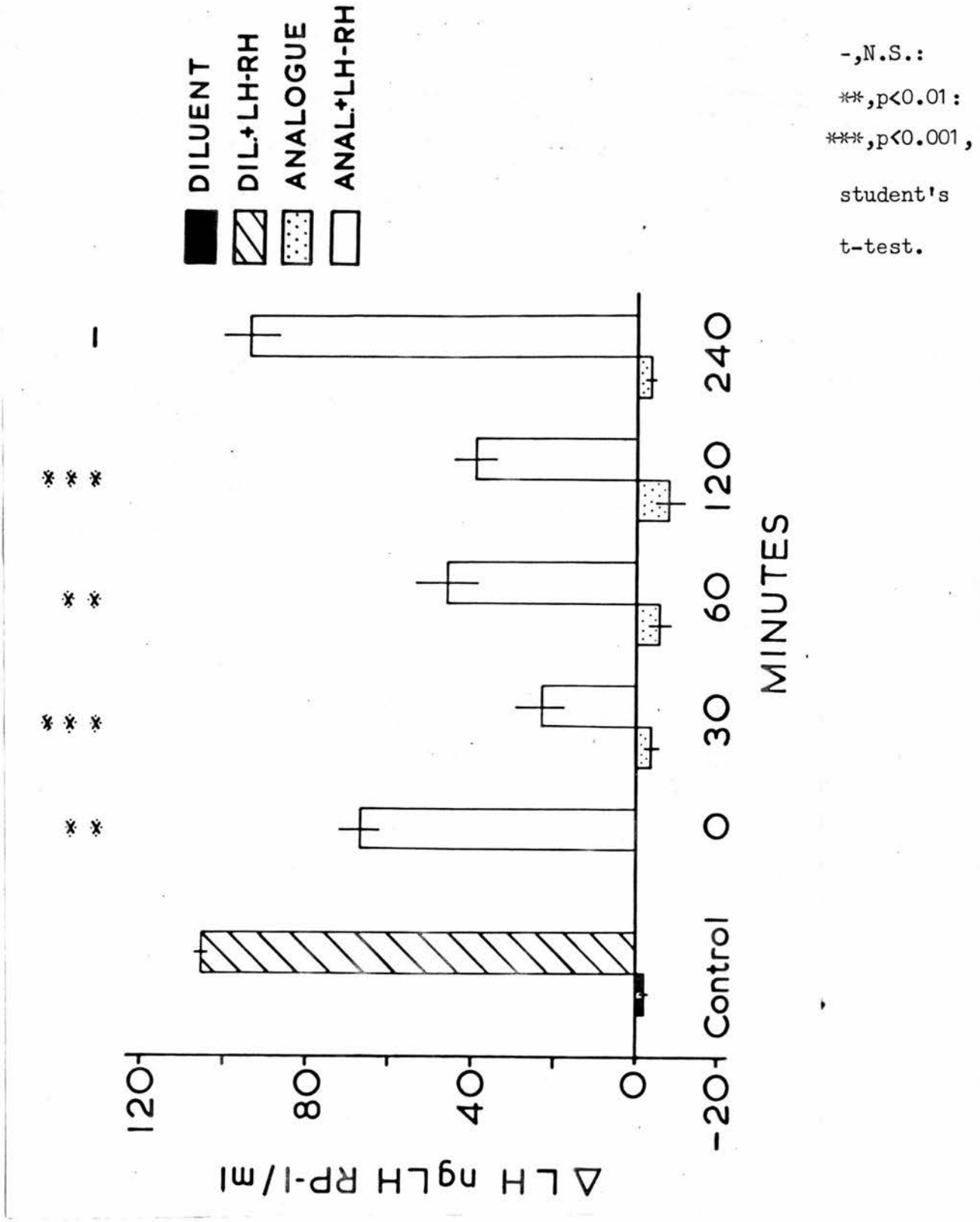


Figure 3.16

Time course in male marmosets of blockade of LH release in response to LH-RH by D Phe^2 D Trp^3 D Phe^6 LH-RH (1.0mg per animal)

Analogue was injected at time 0. Values are mean ± S.E.M.



30 min after the injection (see also chapter 4). Both analogues suppressed LH-RH induced LH release although not to the same extent. Peptide A significantly suppressed LH release 30, 60, 120 and 240 min after administration, with the greatest suppression (71.9%) occurring after 60 min. There was no significant difference between LH-RH induced LH release in the absence of analogue and after simultaneous administration of LH-RH and analogue. Peptide B significantly suppressed LH release after 0, 30, 60 and 120 min, but not after 240 min. Maximum suppression, which occurred after 30 min, was 78.1%. Both peptides had a slight, but non-significant ($p > 0.05$, students t-test) inhibitory action on tonic LH secretion, and no effect of the diluent could be observed.

3.4 Discussion

3.4.a Immunisation against LH-RH

The present study describes the effects of active immunisation against LH-RH in male and female marmoset monkeys. The results demonstrate that in the marmoset, as shown previously in the rat (Fraser, Gunn, Jeffcoate and Holland, 1974a; Fraser, Jeffcoate, Gunn and Holland, 1975) and rabbit (Fraser, 1975), the action of LH-RH can be inhibited by active immunisation. Since antibodies to the synthetic decapeptide cross-reacted with endogenous LH-RH, it is possible that the natural marmoset releasing hormone is also a decapeptide, with a structure similar to that of the synthetic preparation used (Matsuo et al, 1971). Although there is no direct evidence to show that immunisation against LH-RH inhibits LH and FSH secretion in the marmoset, this is clearly the case in the rat (Fraser et al, 1974a; Fraser, 1975), and it is inconceivable that the suppression of gonadal

activity described in antibody producing marmosets is not causally related to a suppression of gonadotrophin secretion.

The immunogen used in this study, LH-RH conjugated to BSA by carbodiimide (Fraser, Gunn, Jeffcoate and Holland, 1974b) was effective in inducing LH-RH antibody formation in all animals, although to a much lesser extent in four animals than in the remainder. Since LH-RH antibodies were not produced by immunisation against BSA, it is reasonable to assume that the antibodies present in the circulation of LH-RH immunised animals were produced specifically in response to the LH-RH immunogen. The usefulness of booster injections is difficult to assess from the present study since the first booster caused an accelerated production of antibodies in only some of the animals, and a second booster in those animals which received one, appeared to have little effect. Whether a primary immunisation alone is sufficient to effectively inhibit LH-RH action in the marmoset, as it appears to be in rodents (Fraser, 1975) remains to be determined. The time at which antibodies to LH-RH first appear in the circulation of the marmoset (3-5 weeks after immunisation in high titre animals) is similar to that in rats (about 3 weeks, Fraser et al, 1974a). Three of the 10 animals immunised against LH-RH died during the course of this study. Autopsy showed that ♂4 and ♀ 3 died of a respiratory infection which is unlikely to have been due to the presence of LH-RH antibodies since other (non-immunised) animals in the colony were also affected. The cause of death of ♀ 4 is not clear although the animal was in poor condition as a result of extensive dermal lesions at the immunisation sites. There were, however, no obvious abnormalities in any of the major abdominal or thoracic organs.

The observed pattern of testosterone secretion is clearly different in high titre animals compared with either low titre or control animals. However the precise nature of testosterone secretion is not revealed by the sampling frequency used. Testosterone secretion in normal male marmosets is subject to considerable short term fluctuations (J. P. Hearn and D. H. Abbott, pers. comm.). The present data do not provide a quantitative account of testosterone production, but merely indicate that elevated testosterone concentrations were not observed during the periods of high antibody titre. Although this would strongly suggest that immunisation against LH-RH suppresses testosterone secretion, thus agreeing with results in the rat (Fraser et al, 1974a; Fraser, 1976b), the possibility that testosterone secretion in the presence of high levels of antibody was not as consistently low as the present data imply must be considered. Furthermore, it is not possible to determine whether testosterone secretion in males with low antibody titres has been suppressed compared with controls. It would be of interest to know this in view of the slight, but obvious decrease in testis volume seen in animals with low antibody titres.

Both macroscopic and microscopic atrophy of the testes was seen in all LH-RH immunised animals, and was related to the levels of circulating antibody. The marked atrophication of the testes in high titre animals was associated with severe impairment of spermatogenesis, as revealed by histological examination of testes removed 53 or 67 weeks after immunisation. The tubules contained spermatogonia and spermatocytes, but there was a marked absence of spermatozoa. In addition, a reduction in the amount of interstitial tissue suggests that steroidogenesis was inhibited and thus supports the results on

plasma testosterone concentrations. Similar effects of high levels of LH-RH antibodies on testicular morphology have been previously reported in rodents (Fraser et al, 1974a; Fraser, 1975).

In female marmosets, high levels of circulating antibody were associated with an absence of ovulatory cycles, as evidenced by low circulating levels of progesterone (with the exception of ♀ 1 before abortion). Several investigators have reported that both active (Fraser, 1975; 1976b)and passive (Koch, Chobsieng, Zor, Fridkin and Lindner, 1973; Arimura, Debeljuk and Schally, 1974; Kerdelhue et al, 1975) immunisation against LH-RH in the rat will also prevent ovulation. It should however be pointed out that since the luteal phase in the normal marmoset cycle is approximately 10 days long (Hearn and Lunn, 1975), the presence of a functional corpora lutea may have been missed during the period when blood samples were collected at 5-10 week intervals. The effects of immunisation against LH-RH on ovulation in the marmoset are complicated by the fact that anovulatory cycles also occurred in at least one female with low antibody titres (♀ 3, and perhaps also ♀ 2, 12-50 weeks after immunisation). Low levels of antibody may, in fact, be capable of preventing ovulation but other factors, particularly stress imposed by experimental procedures, should also be considered.

Ovarian histology revealed the presence of luteal tissue in control but not in LH-RH immunised animals. Since the luteal phase in the marmoset is much longer than the follicular phase, it is relatively difficult to obtain ovaries completely devoid of luteal tissue. The fact that none of the ovaries from immunised animals contained luteal tissue therefore provides further evidence that ovulation in these animals had ceased. Despite the absence of

ovulation, animals with low antibody titres showed continuous follicular growth to the antral stage. It is unlikely that the follicular growth was stimulated by the LH-RH-BSA conjugate itself, since it has been shown to be devoid of LH-releasing activity (Sandow, Von Rechenberg, Wissmann, Uhman and Fraser, 1977). It is therefore probable that the inhibition of LH-RH by low levels of antibody was incomplete, and that follicular growth occurred in response to low gonadotrophin levels which were insufficient to cause ovulation. A more complete inhibition of LH-RH (with corresponding lower gonadotrophin levels) in animals with high antibody titres is suggested by the absence of follicular development beyond the pre-antral stage, and the marked reduction in interstitial tissue in these animals. Measurement of circulating LH and FSH concentrations in immunised marmosets is necessary to confirm this.

Pituitary function in immunised animals has been tested by the administration of exogenous LH-RH. Whereas an apparently normal response to the 4ug dose was observed in low titre animals, LH release was greatly reduced in high titre animals, and even with a massive dose of LH-RH (50ug), pituitary response was considerably diminished. Nevertheless there was a certain amount of LH release, showing that even in animals with high antibody titres that had been immunised for approximately 40 weeks and were apparently infertile, the pituitary gonadotrophs were still able to respond to the releasing hormone. LH release by pituitary gonadotrophs following LH-RH immunisation has also been demonstrated by administration of high doses of LH-RH in rats (Fraser, 1976b). A much greater gonadotrophin release in LH-RH immunised rats has recently been reported using a highly active analogue of LH-RH (Fraser and Sandow, 1977). In

addition to having prolonged LH-RH activity the analogue is immunologically different from LH-RH and can stimulate gonadotrophin release in immunised animals without interference from the antibody. The use of such potent LH-RH analogues may be potentially useful for reversing the effects of immunisation against LH-RH.

Reversability of the effects of immunisation against LH-RH is an important consideration in evaluating the potential of such a procedure as a possible form of fertility control. Although information on reversibility in the present study is incomplete, a natural reversal following decrease in antibody titres can be seen in some of the animals. Two immunised females (♀ 1, ♀ 2) became pregnant and gave birth to viable offspring, although in one case (♀ 2), immunisation produced only low levels of antibody. Both of the high titre males which survived to the end of the study (♂1, ♂5) showed clear signs of increased testicular activity following the decline in antibody titres. In all cases signs of reversal appeared at least 50 weeks after primary immunisation. This period is longer than that reported by Fraser (1976b)in which signs of reversal appeared 30-34 weeks after immunisation in male rats, although in this case booster injections were not given.

3.4.b Inhibition of LH-RH with inactive analogues

LH release in response to exogenous LH-RH was inhibited by both analogues tested, but not by the diluent. It is therefore reasonable to assume that the inhibition was due to a direct antagonism between the peptides themselves and the synthetic releasing hormone. The exact mechanism by which the analogues exert their effect is not completely understood, although it is generally assumed that they bind to pituitary receptor sites, thus competing with LH-RH

and preventing its action (Ferland, Labrie, Coy, Coy and Schally, 1975; Nishi, Coy, Coy, Arimura and Schally, 1976). Since the structures of many of these analogues are based on super-active analogues, they often possess residual inherent LH-RH activities (Vilchez-Martinez, Schally, Coy, Coy, Debeljuk and Arimura, 1974). However, de la Cruz et al, (1976) have reported that D Phe^2 Phe^3 D Phe^6 LH-RH (A) was devoid of any such releasing activity when tested in immature male rats. The present results confirm this observation and also indicate a similar absence of LH releasing activity in D Phe^2 D Trp^3 D Phe^6 LH-RH (B).

The data presented here also agree with previous observations in rats (de la Cruz et al, 1976; Nishi et al, 1976) that the peak antagonist activity of peptide A was between 30 and 120 min. The effects of peptide B have not previously been reported. The present results are insufficient to determine whether there is any significant difference between the activities of the two analogues tested but they do suggest that peptide B may cause a more rapid inhibition of LH release than peptide A. The greatest suppression of LH release by peptide A (71.9%) is lower than that (92.6%) reported by de la Cruz et al (1976) in rats. This discrepancy may be explained by the fact that in the present study the analogue could not be completely dissolved before use. A suspension would presumably not be absorbed as rapidly or as effectively as an homogeneous solution. In addition, the analogue:LH-RH ratio used by de la Cruz et al was greater (2500:1) than in the present study (2000:1).

3.5 Chapter summary

1. Antibodies to LH-RH can be raised in the marmoset by active immunisation against a synthetic LH-RH-BSA conjugate. All LH-RH immunised animals produced antibodies, although in four of the animals titres remained low.

2. In males, high levels of antibody produced testicular atrophy and a suppression of testosterone secretion. Seminiferous tubules were reduced in size, spermatogenesis was severely impaired, and there was a reduction in the amount of interstitial tissue.

3. Although there was slight reduction in testis volume and seminiferous tubule diameter in males with low antibody titres, testosterone secretion and spermatogenesis appeared normal.

4. Progesterone concentrations in females with high antibody titres suggest that ovulation had ceased. Ovaries were reduced in size compared with controls and contained many small pre-antral follicles but no luteal tissue.

5. Anovulatory cycles were also apparent in females with low antibody titres. The ovaries were also reduced in size and no luteal tissue could be seen. However, ovarian follicles in these animals developed to a later stage than in animals with high antibody titres, suggesting a less complete inhibition of gonadotrophin secretion.

6. Pituitary LH release in response to exogenous LH-RH was inhibited in animals with high antibody titres, but apparently not in animals with low antibody titres.

7. Although the data are incomplete, the effects of immunisation against LH-RH in the marmoset appear to be reversible.

8. Antagonistic analogues of LH-RH inhibited LH release in response to exogenous LH-RH, with maximum inhibition occurring after 30-60 min.

CHAPTER FOUR

PITUITARY RESPONSIVENESS TO LH-RH AND THE INFLUENCE OF GONADAL STEROIDS

4.1 Introduction

Secretion of LH by the pituitary gland is directly related to hypothalamic LH-RH stimulation. Administration of exogenous (synthetic) LH-RH has provided the basis for numerous studies on the characteristics of LH-RH induced LH release, and it is now apparent that the decapeptide described by Matsuo et al (1971) is effective in stimulating LH secretion in a variety of mammalian species including rats (e.g. Schally, Arimura and Kastin, 1973), sheep (e.g. Reeves, Arimura and Schally, 1971), rhesus monkeys (e.g. Krey et al, 1973) and humans (e.g. Yen et al, 1975a).

The secretion of LH in response to LH-RH is, however, profoundly modified by the action of gonadal steroids, and it is now known that at least part of this action is focussed directly on the hypophysis and is reflected in changes in the sensitivity of the gonadotrophs to LH-RH stimulation. Pre-treatment with gonadal steroids (primarily oestradiol, but also progesterone) modifies the pituitary response to a standard dose of LH-RH in the rhesus monkey (Krey et al, 1973) and human (Lasley et al, 1975; Young and Jaffe, 1976), as well as in rats and sheep (Debeljuk, Arimura and Schally, 1972b). Steroid induced changes in pituitary responsiveness to LH-RH, responsible for the variations in LH release to LH-RH during different phases of the menstrual cycle (Krey et al, 1973; Yen et al, 1975a), therefore represent an important mechanism by which the secretion of LH is regulated by steroid feedback.

The present study describes the LH response to exogenous LH-RH in the marmoset monkey, and looks at some of the ways in which this response can be modified by the action of gonadal steroids.

4.2 Procedure

Groups of intact male marmosets (n=4 per group) were each given single rapid intramuscular injections of one of the following doses of LH-RH in 0.1 ml saline : 0.2, 0.5, 1, 2, 5, 10, 25 ug. Six intact males received saline alone. Blood samples were taken immediately before and 30, 45, 60 and 90 minutes after each injection. In addition four intact males were given 25 ug LH-RH in 0.1 ml saline (i.m.) and were bled immediately before and 10, 20, 30 and 90 min. after each injection. Plasma LH concentrations were measured in all blood samples.

Pituitary responsiveness to a single rapid injection (i.m.) of 2.0 ug LH-RH in 0.1 ml saline was studied in the following groups of animals:

Group 1. Intact males (n=18).

Group 2. Gonadectomised marmosets 48h (4 males; 4 females) and approximately 16 weeks (10 males; 6 females) after bilateral gonadectomy.

Group 3. Long term gonadectomised marmosets 6 days (5 males; 5 females) and 3 weeks (5 males; 4 females) after the insertion of oestradiol-17β implants.

Group 4. Long term gonadectomised marmosets (4 males; 4 females) 6 days after the insertion of progesterone implants.

Blood samples collected immediately before, and 30, 45, 60 and 90 min after each injection were assayed for LH. Oestradiol-17β and progesterone were measured immediately before the first injection of LH-RH.

Due to differences in pre-injection LH concentrations between various groups, pituitary responsiveness was measured as the increase in LH levels above pre-injection levels (ΔLH), as well as in terms of absolute concentrations.

4.3 Results

4.3.a Response to various doses of LH-RH

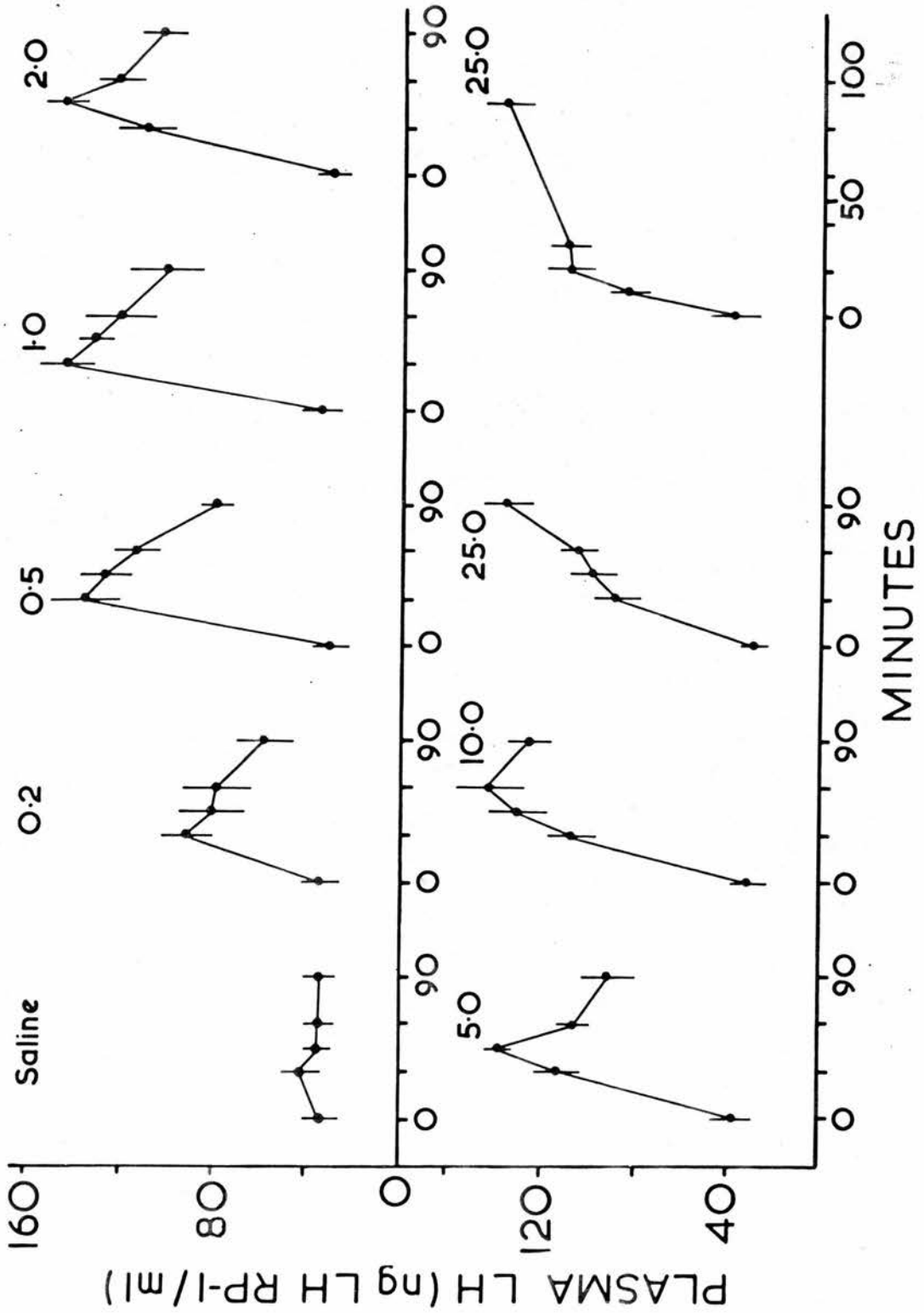
Rapid increases in plasma LH concentrations were measured in all animals receiving LH-RH, but not in the animals that were given saline alone (Fig. 4.1). Maximum LH concentrations achieved by 0.5-25 ug doses of LH-RH were not significantly different from each other, but they were all significantly higher compared with maximum LH levels produced by 0.2 ug LH-RH ($p < 0.05$, students t-test). With all doses used there was a significant increase in plasma LH concentrations by 30 min. after the injections ($p < 0.05$, paired t-test), although the time at which peak levels of LH occurred became progressively later as the dosage increased. Thus, maximum LH concentrations were measured after 30 min. when 0.2, 0.5 and 1.0 ug LH-RH were administered, after 45 min. with 2 and 5 ug LH-RH, after 60 min.with 10 ug LH-RH, and after 90 min. with 25 ug LH-RH. Blood samples taken at 10 min. intervals for 30 min. after the administrations of 25 ug LH-RH (Fig. 4.1) failed to reveal any significant peak in LH secretion during this period.

4.3.b The effects of gonadectomy and steroid treatment on pituitary responsiveness to LH-RH

From the results shown in Figure 4.1, 2 ug LH-RH was chosen as the dose to be used in all subsequent LH-RH tests.

Figure 4.1

Mean (± S.E.M.) plasma LH concentrations following i.m. injection of saline (n=6) and various doses of LH-RH (n=4 per dose) to intact males



Group 1.

Mean ($\pm$ S.E.M.) LH concentrations in intact males following administration of LH-RH or saline are shown in Figure 4.2. The hatched area represents the range of LH concentrations measured, and indicates considerable variation in the response to a standard dose of LH-RH. From the sampling frequency used, it appears that plasma LH concentrations reach a maximum after 45 min. and remain elevated until at least 90 min. after the injection. Mean LH concentrations were significantly higher than mean control values at all times after the injection ($p < 0.01$, students t-test).

Group 2.

Mean ($\pm$S.E.M.) plasma LH concentrations following administration of LH-RH to male and female gonadectomised marmosets are shown in Figures 4.3 and 4.5 respectively. LH values for both males and females show a significantly greater response at all times in animals gonadectomised for 16 weeks (long term) compared with animals gonadectomised for 2 days (short term) (Figs. 4.4 and 4.6). Responses in males and females were not significantly different ($p < 0.05$, student's t-test). Compared with intact males, ΔLH values were significantly greater in long term, but not in short term gonadectomised males.

Group 3.

The introduction of oestradiol-17β implants into long term gonadectomised marmosets inhibited pituitary responsiveness to LH-RH. Plasma LH concentrations following an injection of LH-RH in gonadectomised animals containing implants for either 6 days or 3 weeks are shown in Figures 4.7 and 4.9. There was no difference between circulating levels of oestradiol-17β 6 days and 3 weeks after insertion

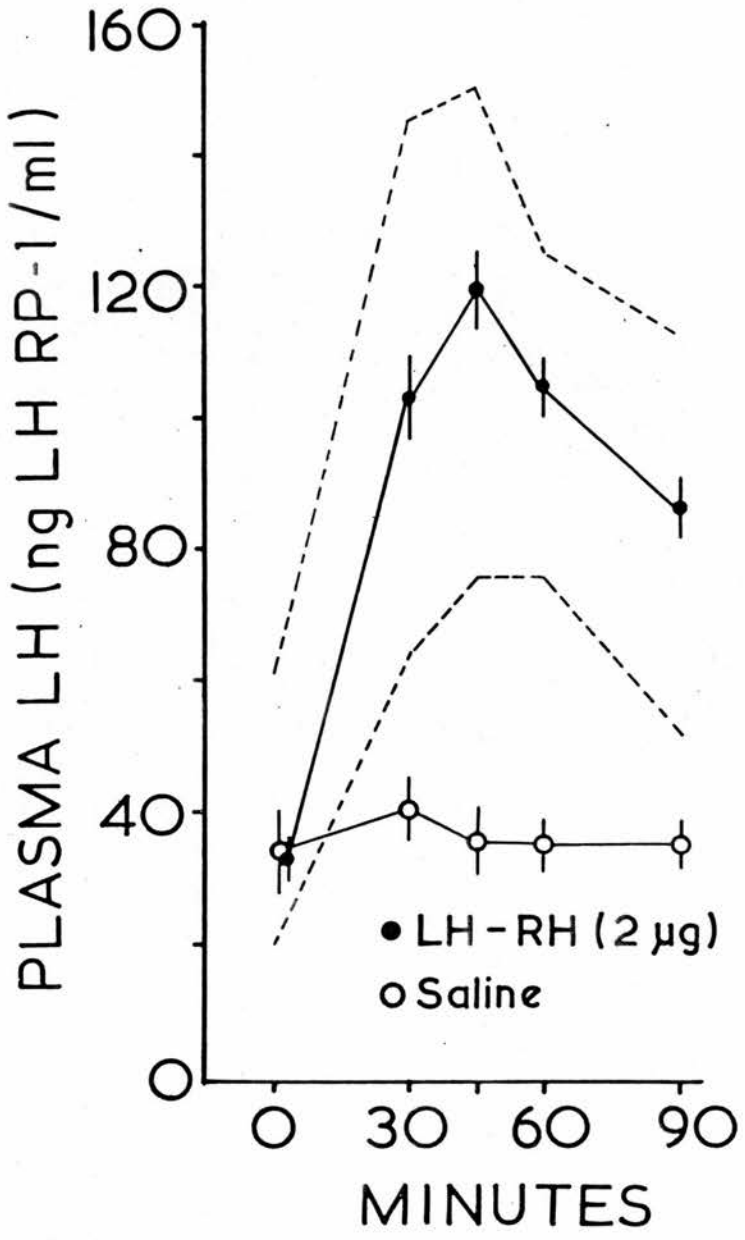


Figure 4.2

Response to a single i.m. injection of 2ug LH-RH (n=18), or saline (n=6) in intact males

Values are mean ± S.E.M., with range for animals receiving LH-RH

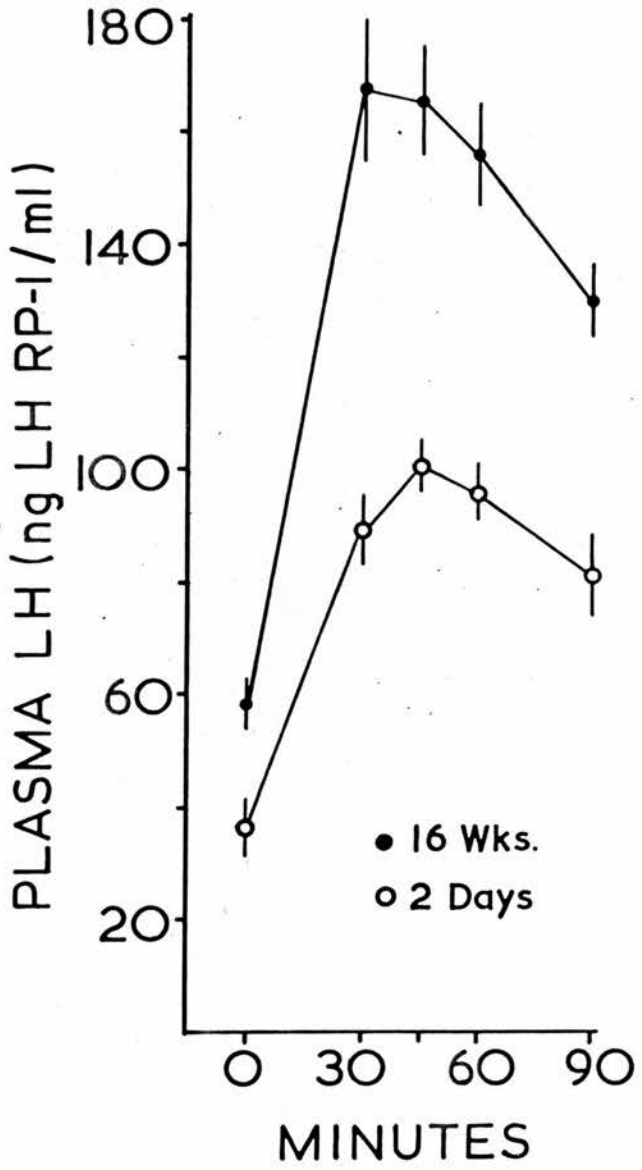


Figure 4.3

Response to a single i.m. injection of 2ug LH-RH in males gonadectomised for 2 days (n=4) and 16 weeks (n=10)

Values are mean ± S.E.M.

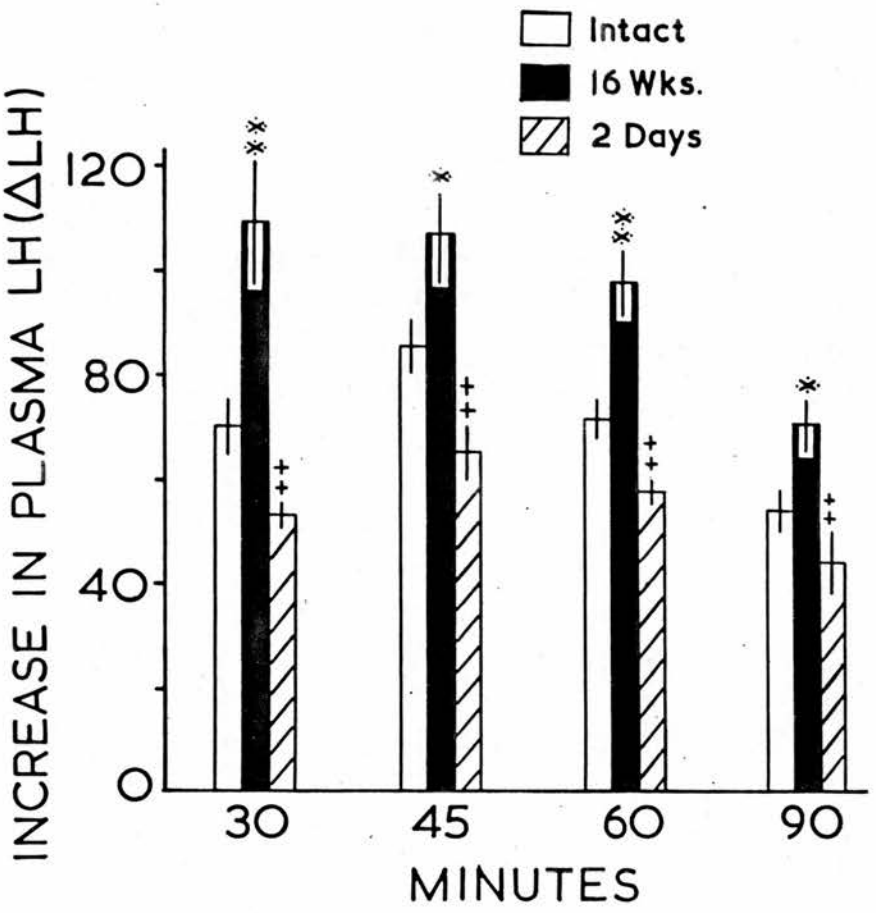


Figure 4.4

Increase in LH concentrations following 2ug LH-RH in intact males (n=18) (A) and males gonadectomised for 2 days (n=4) (B) and 16 weeks (n=10) (C)

Mean values $\pm$ S.E.M.

* A vs C : + B vs C : A vs B not significant.

For this figure, and for figures 4.6, 4.8, 4.10, 4.12 and 4.13 the levels of significance are indicated;

3 symbols (i.e. ***), $p < 0.001$: 2 symbols (i.e. **), $p < 0.01$:

1 symbol (i.e. *), $p < 0.05$. (Student's t-test).

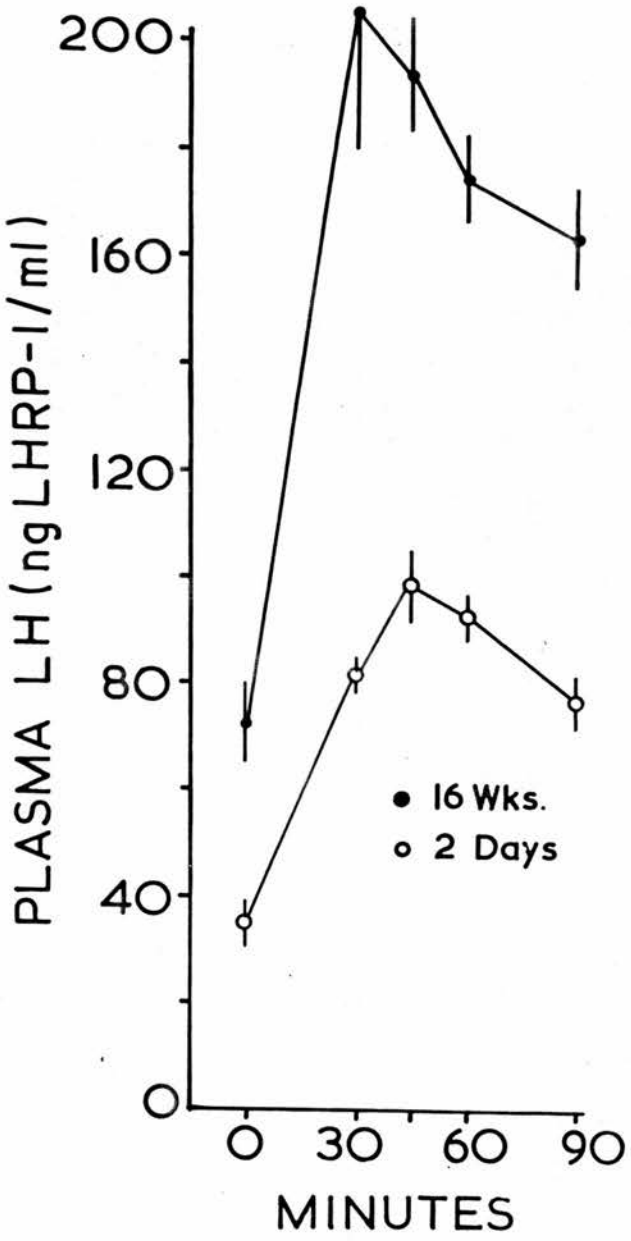


Figure 4.5

Response to a single i.m. injection of 2ug LH-RH in females gonadectomised for 2 days (n=4) and 16 weeks (n=6)

Values are Mean $\pm$ S.E.M.

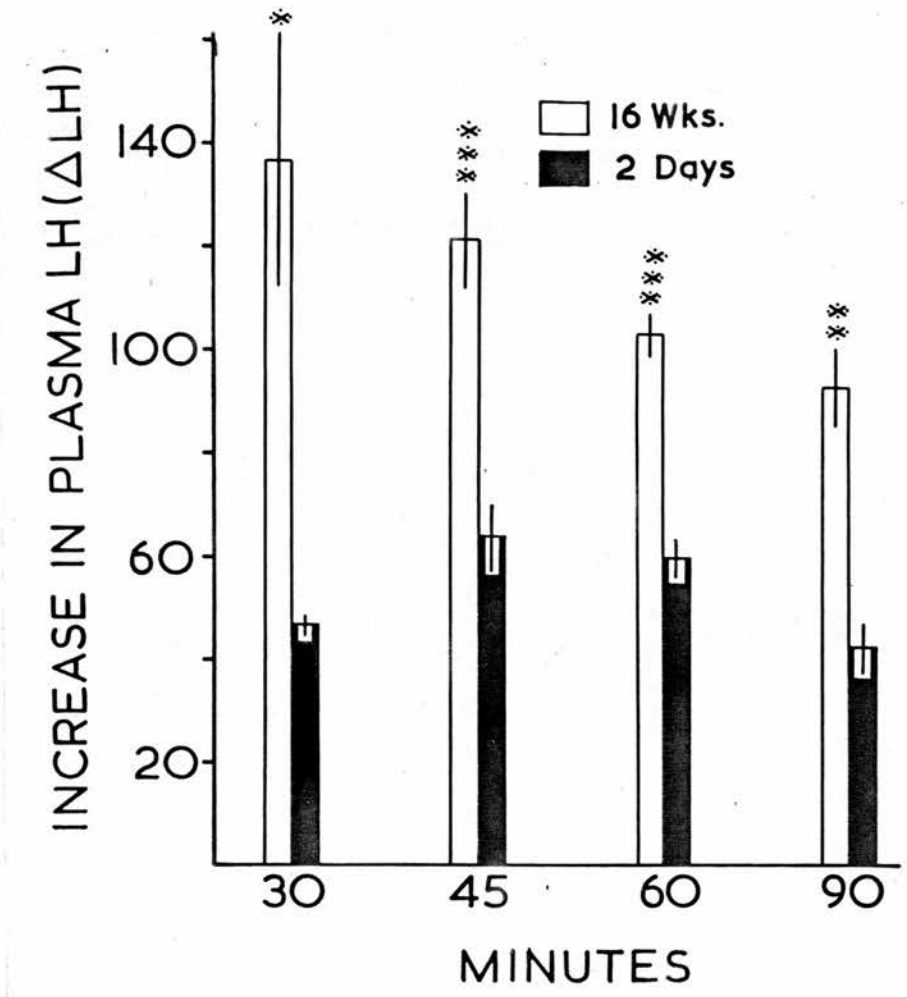


Figure 4.6

Increase in LH concentrations following 2ug LH-RH in females gonadectomised for 2 days (n=4) and 16 weeks (n=6)

Mean values ± S.E.M.

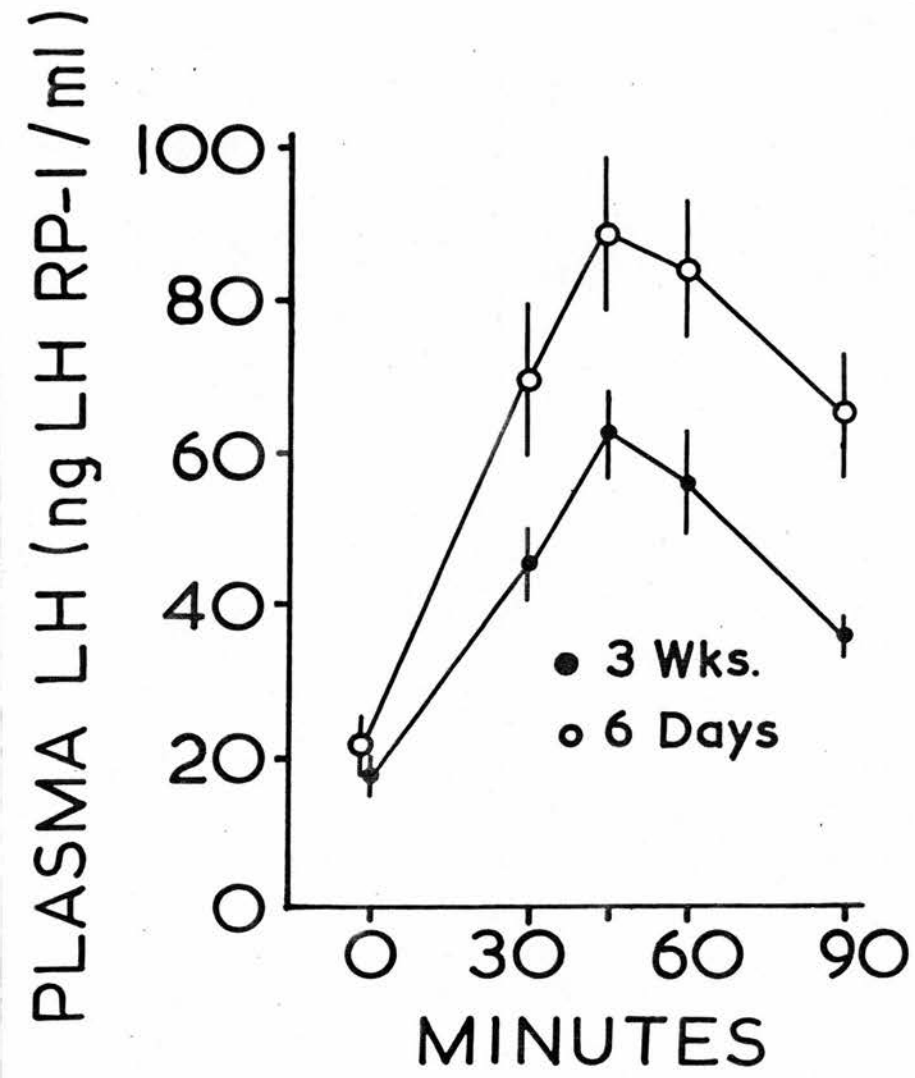


Figure 4.7

Response to a single i.m. injection of 2 ug LH-RH in long term gonadectomised males implanted with oestradiol-17β for 6 days (n=5) and 3 weeks (n=5)

Values are Mean $\pm$ S.E.M.

of the implants. The levels have therefore been combined to produce mean (±S.E.M.) values of 0.56 ± 0.03 ng/ml (males, n=10) and 0.54 ± 0.04 ng/ml (females, n=9). In terms of ΔLH values, the pituitary response was significantly lower in implanted animals (both short and long term) when compared with gonadectomised non-implanted animals (Figs. 4.8 and 4.10). Although ΔLH values were significantly lower in long term implanted animals compared with short term implanted animals in males (significant after 30, 60 and 90 mins) but not in females, there were no significant differences between male and female responses ($p > 0.05$, student's t-test, at all times).

Group 4

In contrast to the effects of oestrogen, implants of progesterone appeared to enhance, rather than inhibit pituitary responsiveness to LH-RH in long term gonadectomised animals (Fig. 4.11). Mean (±S.E.M.) progesterone concentrations in males and females achieved by the implants were 36.4 ± 3.3 and 42.7 ± 5.2 ng/ml respectively. LH values in progesterone implanted animals were significantly elevated compared with non-implanted animals after 30, 45 and 60 min. (males), and after 45 and 60 min. (females) (Figures 4.12 and 4.13). There were no significant differences between male and female responses ($p > 0.05$, student's t-test, at all times).

4.4 Discussion

All the intact male marmosets that received LH-RH, regardless of the dosage used, showed a marked increase in circulating LH concentrations. As a similar release of LH was not seen in animals

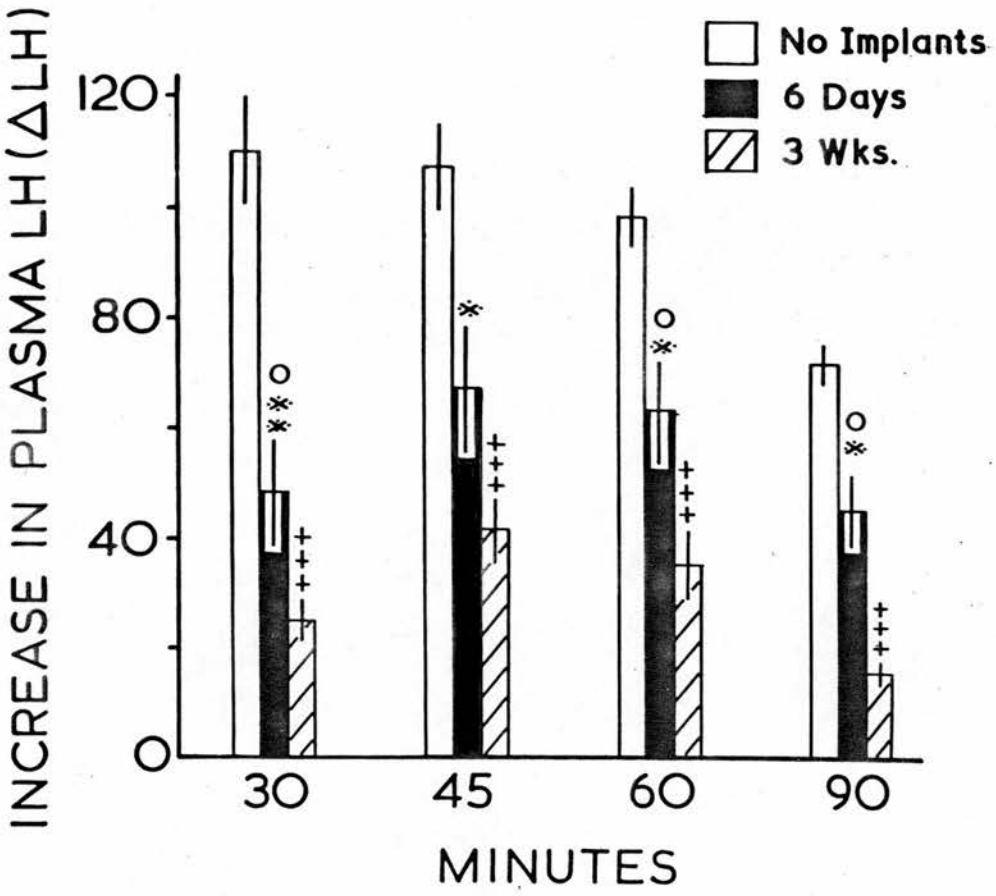


Figure 4.8

Increase in LH concentrations following 2ug LH-RH in long term gonadectomised males not implanted (n=10) (A), and implanted with oestradiol-17β for 6 days (n=5) (B) and 3 weeks (n=5) (C)

Mean values $\pm$ S.E.M.

* A vs B

\+ A vs C

o B vs C

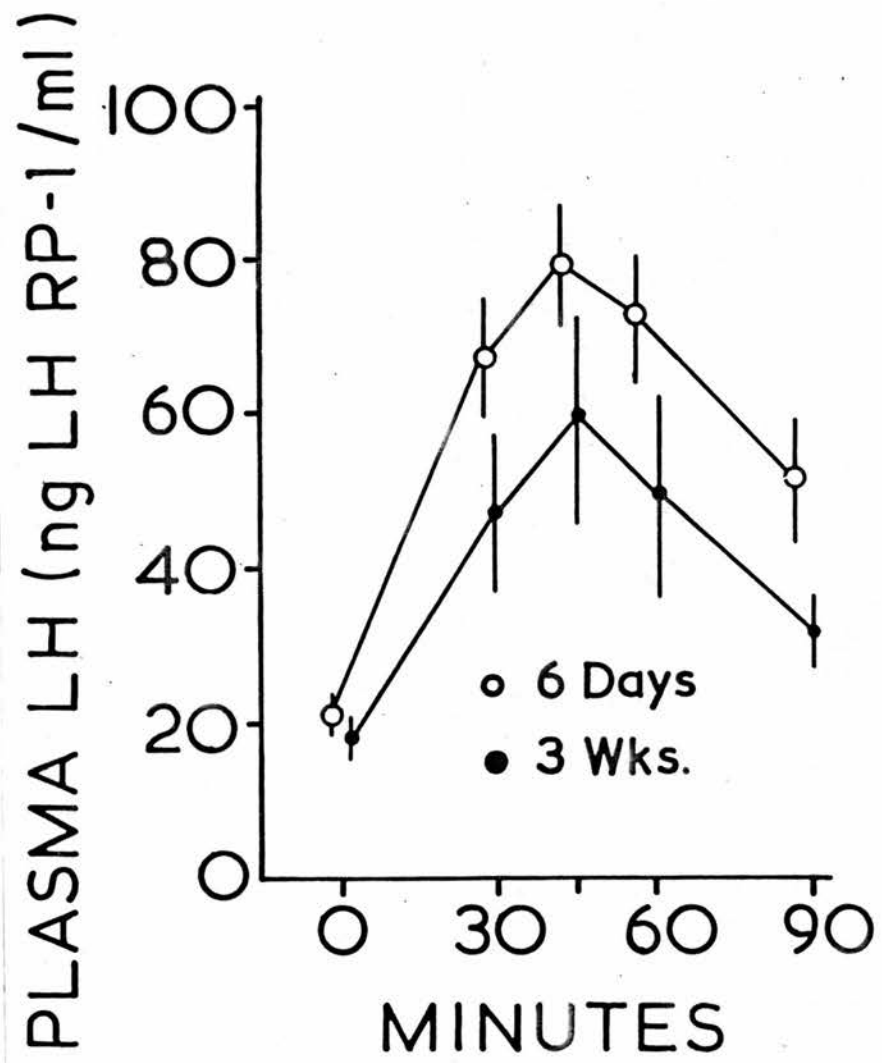


Figure 4.9

Response to a single i.m. injection of 2ug LH-RH in long term gonadectomised females implanted with oestradiol-17β for 6 days (n=5) and 3 weeks (n=4)

Values are Mean ± S.E.M.

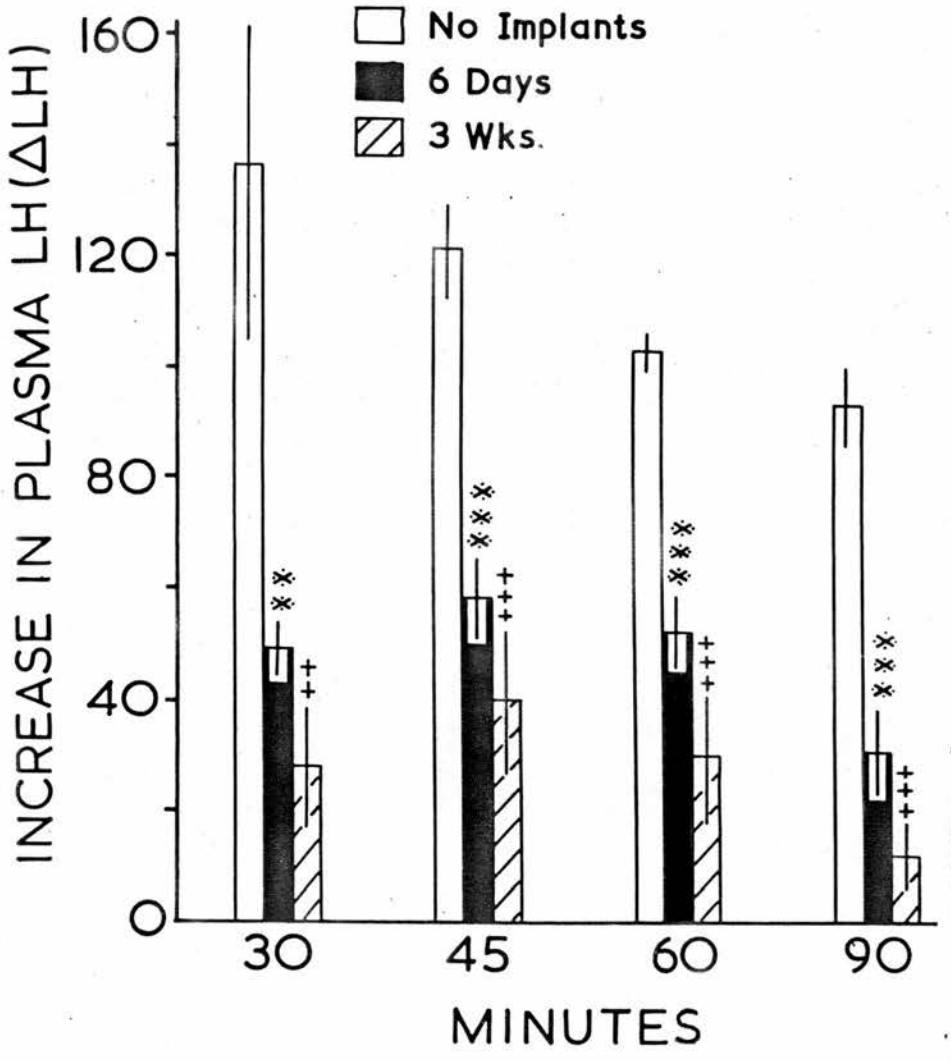


Figure 4.10

Increase in LH concentrations following 2ug LH-RH in long term gonadectomised females not implanted (n=6) (A), and implanted with oestradiol-17β for 6 days (n=5) (B) and 3 weeks (n=4) (C)

Mean values $\pm$ S.E.M.

* A vs B

\+ A vs C

(B vs C, not significant)

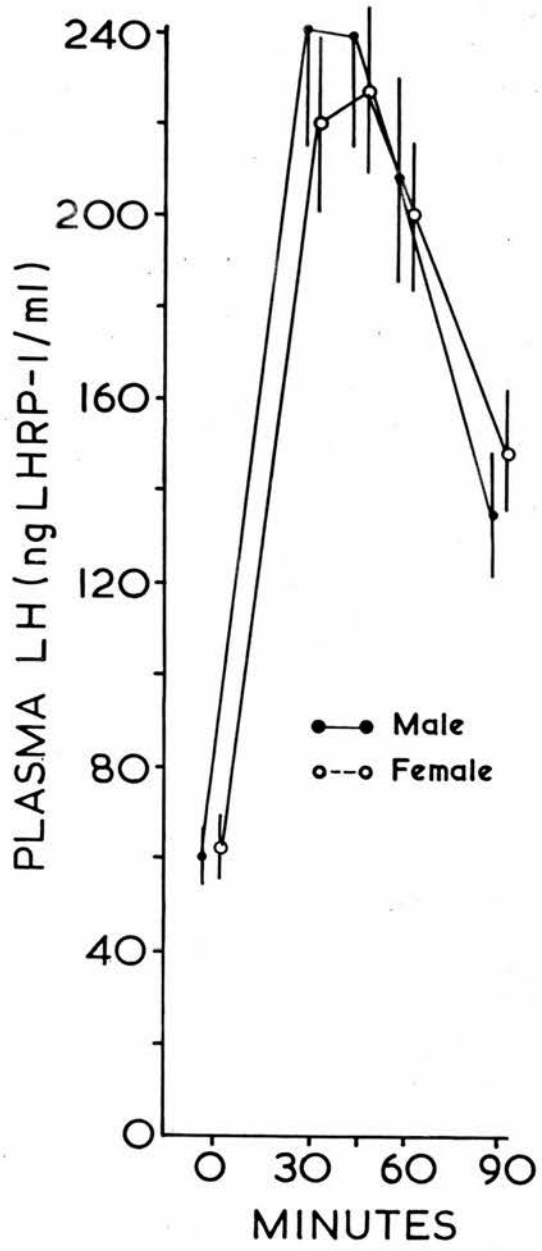


Figure 4.11

Response to a single i.m. injection of 2ug LH-RH in long term gonadectomised males (n=4) and females (n=4) implanted with progesterone for 6 days

Values are Mean ± S.E.M.

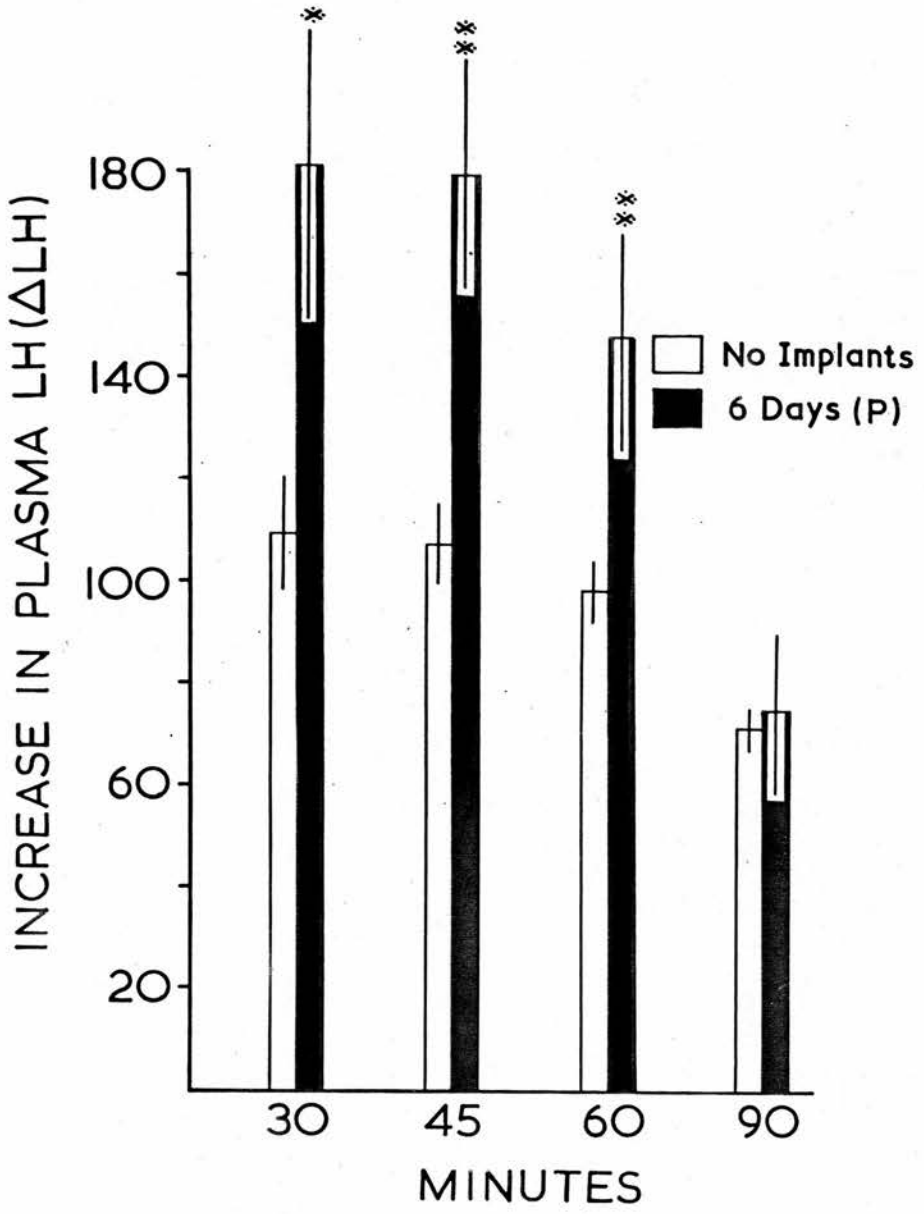


Figure 4.12

Increase in LH concentrations following 2ug LH-RH in long term gonadectomised males not implanted (n=10), and implanted with progesterone for 6 days (n=4)

Mean values ± S.E.M.

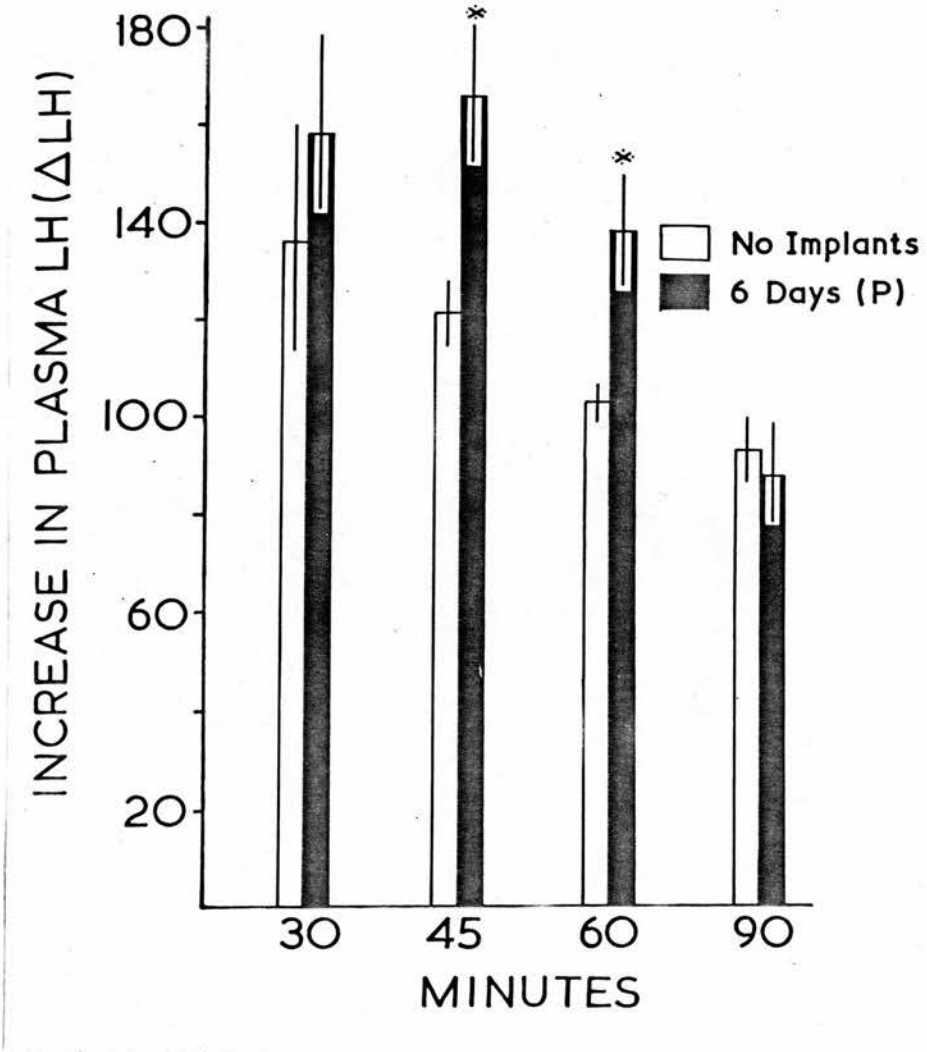


Figure 4.13

Increase in LH concentrations following 2ug LH-RH in long term gonadectomised females not implanted (n=6), and implanted with progesterone for 6 days (n=4)

Mean values $\pm$ S.E.M.

that received saline, it is apparent that the responses of the LH-RH treated marmosets were primarily the result of the injection of releasing hormone. These observations support the conclusions drawn from the immunisation study (Chapter 3) that the natural marmoset releasing hormone may be a decapeptide with a structure at least similar to that of the synthetic preparation used (Matsuo et al, 1971).

Dose response characteristics shown in Figure 4.1 revealed little difference between the maximum LH levels achieved in response to doses of LH-RH ranging from 0.5-25ug. It is possible that the 0.5 ug dose induced a maximum response by the pituitary and that further increments in the dose of LH-RH would have no further effect on LH secretion. This eventuality, however, is not supported by the change in the pattern of LH release, which is clearly taking place as the dose of LH-RH increases. A more likely explanation may be that since the frequency and duration of sampling were insufficient to observe the complete profile of LH-RH induced LH release, other differences between the responses have not been revealed. In this respect, when Krey et al (1973) tested increasing doses of LH-RH in intact female rhesus monkeys, they measured little difference in the LH response over the first hour, but found that LH levels remained elevated for progressively greater periods of time as the dose increased. More recent work in humans (Wang et al, 1976; Hoff et al, 1977) has demonstrated the presence of two functionally separable pools of pituitary LH, one immediately releasable (pool one, reflecting sensitivity) and the other requiring continued stimulus input (pool two, reflecting capacity or reserve). Thus, whereas only the immediately releasable pool of LH is affected by low doses of LH-RH, larger doses may provide sufficient stimulus to evoke LH release from

both pools. This may help to explain the delay in appearance of peak LH concentrations seen with the higher doses of LH-RH in the present study, but does not account for the apparent differences in the initial rate of LH secretion. The present results do nevertheless show that a wide range of doses of LH-RH will evoke a marked increase in pituitary LH secretion in the marmoset monkey. Although the decision to use 2 ug LH-RH for the rest of the study was somewhat arbitrary, it would not seem unreasonable in view of the (limited) information presented in Figure 4.1.

Comparison of the responses to LH-RH between intact and long term gonadectomised males shows a clear augmentation of pituitary responsiveness in the hypogonadal state. These results are in agreement with observations in women (Siler and Yen, 1973) in which a greater response to a single injection of LH-RH is obtained in patients with hypogonadal function (gonadal dysgenesis, postmenopausal, and ovariectomised) than in normal subjects during the early follicular phase. A greater response for LH in patients with gonadal dysgenesis as compared with normal adult subjects has also been demonstrated in men (Roth, Kelch, Kaplan and Grumbach, 1972). The present results suggest that the increase in pituitary responsiveness to LH-RH does not occur immediately after gonadectomy, since LH release in males 2 days after gonadectomy was not significantly different from that in intact animals.

Pituitary responsiveness to LH-RH in intact female marmosets has not been measured, and therefore cannot be directly compared with that in gonadectomised animals. If the variability in LH response in different stages of the cycle is as great in the marmoset as in other primate species (Krey et al, 1973; Yen et al, 1975a)then it is

imperative to know during which stage of the cycle the experiment is being performed. Due to the difficulties involved in obtaining this information in the female marmoset, observations on LH-RH induced LH release in intact animals have been restricted to males. Nevertheless, the differences between short and long term gonadectomised females shown in Figure 4.5 (c.f. males, Fig. 4.3) make it reasonable to assume that pituitary responsiveness to LH-RH is also augmented in females in the absence of gonadal steroids. In agreement with observations in castrated rats (Barraclough and Turgeon, 1975), the results for long and short term gonadectomised marmosets suggest that there is no difference between the sexes in their pituitary responses to LH-RH.

The enhanced LH response in long term gonadectomised marmosets may partially be explained in terms of an increased pituitary LH store, at least the readily releasable component. This interpretation is consistent with the finding of an increased pituitary LH content in postmenopausal women (Ryan, 1962) and in castrated rats (Gay and Hauger, 1977). Furthermore repeated injections of submaximal doses (10 ug) of LH-RH to hypogonadal women (Lasley et al, 1975) have revealed a large pool of acutely releasable LH with a relatively smaller reserve pool, thus indicating a high pituitary sensitivity but a lower capacity. In this context Wang et al (1975) have found that increased doses of LH-RH to hypogonadal women are not able to elicit additional LH release, suggesting that pituitary sensitivity is at a maximum.

The introduction of oestradiol 17β implants into male and female gonadectomised marmosets, either for 6 days or for 3 weeks reduced pituitary responsiveness to LH-RH. All long term implanted

animals showed a smaller LH response than short term implanted animals although, due to considerable inter-animal variation in females, the difference was significant only in males. The levels of circulating oestrogen achieved by the implants are similar to those found during the mid-late follicular phase of the normal female cycle (Hearn and Lunn, 1975). This dose therefore should be considered as being relatively large, and with respect to circulating levels of oestradiol in intact male marmosets, is almost certainly pharmacological.

Studies on the effects of oestrogen on the responsiveness of the anterior pituitary to exogenous LH-RH have been numerous. As might be expected, the results from these studies have revealed a whole spectrum of effects, depending upon the dose and duration of the oestrogen treatment, as well as on the dose and method of LH-RH administration. It must therefore be appreciated that the effects of oestradiol-17β on pituitary response to LH-RH in the marmoset, as shown in the present results, relate only to the experimental conditions described, and that different, and indeed opposite effects can be expected under different circumstances.

Nevertheless, the results clearly indicate that oestrogen can impair pituitary responsiveness to LH-RH in male and female marmosets. Similarly, in the ovariectomised rhesus monkey, oestrogen implants achieving either early or late follicular phase oestradiol-17β levels, when maintained for 13 days, reduced LH release in response to LH-RH. Oestradiol can also impair pituitary responsiveness to a single injection of LH-RH in normal (Keye and Jaffe, 1974) and hypogonadal (Yen, Vandenberg and Siler, 1974) women, and in normal men (D'Agata, Gulizia, Andò, Vitale and Polosa, 1976). Using pulsed delivery of sub-maximal doses of LH-RH (10 ug every 2 h) to hypo-

gonadal women, Lasley et al (1975) have shown that 20 - 50 ug ethinyl oestradiol/day for 7 days induces a functional reversal in the relative activity of the two pools of pituitary LH, with an impediment of sensitivity and a marked augmentation of pituitary reserve. It is therefore possible that the response to a single injection of LH-RH as shown in the marmoset merely represents an impairment in sensitivity, and that pituitary reserve, had it been measured, may have been augmented.

With respect to the difference between LH responsiveness in short term and long term implanted marmosets, Yen, Vandenberg and Siler (1974) have demonstrated in hypogonadal women that chronic treatment with low doses of oestrogen result in an initial enhancement (after 1 and 2 weeks) followed by a progressive diminution of LH-RH induced LH release. If a lower dose of oestrogen had been used in the present study, a more striking difference between short term and long term effects may have been observed.

Circulating progesterone concentrations, maintained at mid luteal phase levels (Hearn and Lunn, 1975) for 6 days appeared to enhance, rather than inhibit pituitary responsiveness to LH-RH in both male and female gonadectomised marmosets. However, the period of enhancement appears to be relatively transient, since the differences between implanted and non-implanted animals had disappeared by 90 min after the LH-RH injection.

That physiological levels of progesterone do not impair LH-RH induced LH release is also suggested by studies in the rhesus monkey (Krey et al, 1973) and women (Wang et al, 1976; Hoff et al, 1977), in which pituitary responsiveness to LH-RH is not diminished during

the mid luteal phase when compared with the early follicular phase of the menstrual cycle. Furthermore, Lasley et al (1975) have shown in women that under conditions in which oestrogen augments LH-RH induced LH release, progesterone, even in relatively low doses, will produce a further amplification in pituitary responsiveness. Although in this case progesterone is acting in combination with oestrogen, the results do lend some support to the present findings in the marmoset. In addition, Martin et al (1974) have shown that advances of spontaneous ovulation by progesterone in rats are accompanied by a corresponding augmentation of pituitary responsiveness to LH-RH. The physiological significance of enhanced pituitary responsiveness to LH-RH by progesterone in male marmosets is not clear.

4.5 Chapter summary

1. Intramuscular administration of synthetic LH-RH stimulates pituitary LH secretion in the marmoset. Although LH release can be induced by a wide range of doses of LH-RH (0.2 - 25ug), peak LH levels appear to be reached progressively later as the dose increases. This may reflect the presence of two functionally separable pools of pituitary LH.

2. Pituitary responsiveness to LH-RH was enhanced by gonadectomy (in the present study direct evidence for this is restricted to males), and is probably the result of an increase in the immediately releasable pool of LH. That the increased pituitary response is likely to reflect a gradual change in the hypothalamic-pituitary system is suggested by the fact that LH release in males 2 days after gonadectomy was not different from that in intact animals.

3. Pituitary response to LH-RH in long term gonadectomised males and females was reduced by oestradiol-17β concentrations of 5-600 pg/ml maintained for either 6 days or 3 weeks, and slightly enhanced by progesterone concentrations of approximately 40 ng/ml maintained for 6 days.

4. These results indicate that gonadal steroids can influence LH secretion by a direct action on the pituitary gland. The importance of dose and duration of steroid treatment and the mode of LH-RH administration in determining steroid induced changes in pituitary response to LH-RH should be emphasised.

CHAPTER FIVE

NEGATIVE FEEDBACK CONTROL OF LH SECRETION

5.1 Introduction

The results from Chapter 4 indicate that the functional relationship between hypothalamic LH-RH and pituitary LH secretion is influenced by gonadal steroids. This influence, directed not only at the anterior pituitary but also at the hypothalamus, and possibly other neural centres, forms the basis of what is known as the feedback control of LH secretion. The two components of this feedback mechanism, negative feedback and positive feedback, will be dealt with in this chapter (negative feedback) and in Chapter 6 (positive feedback).

Tonic LH secretion in primates is regulated by a negative feedback loop existing between the gonads and the hypothalamic-hypophysial axis (Knobil, 1974; Yen et al, 1975a). The interruption of this negative feedback loop by gonadectomy results in a marked increase in circulating LH levels in humans (Yen and Tsai, 1971b; Monroe, Jaffe and Midgley, 1972) and rhesus monkeys (Atkinson et al, 1970), indicating that LH secretion is normally suppressed by gonadal hormones. This increase in circulating LH concentrations is now known to be the result of pulsatile discharges of the hormone by the pituitary gland (Knobil, 1974).

From studies in the rhesus monkey (Karsch et al, 1973a; Knobil, 1974) and human (Nillius and Wide, 1971; Wang et al, 1975) it has been clearly demonstrated that oestradiol is extremely effective as an agent of negative feedback in both males and females. The negative feedback actions of progesterone and testosterone however are somewhat confusing and often contradictory when compared to the clearly suppressive effects of oestradiol on LH. Studies in primates suggest that progesterone on its own has no significant inhibitory effect on

LH secretion (Wallach, Root and Garcia, 1970; Karsch et al, 1973a), whereas physiological doses of progesterone appear to exert a negative feedback action in the ewe (Karsch et al, 1975; Foster and Karsch, 1976). Although testosterone is the principal androgen produced by the testes, and therefore the most logical choice for negative feedback control of LH in males, experimental evidence to support this assumption is not convincing (Resko et al, 1977). Much of the work on negative feedback in primates has been done on hypogonadal subjects and, particularly in the rhesus monkey, a great deal of work has been carried out using long term gonadectomised animals. Although a convenient model, the long term gonadectomised animal may not necessarily be an accurate model for determining the negative feedback action of certain steroids.

The present study describes an initial attempt to characterise some of the relationships between gonadal function and the secretion of LH in the marmoset monkey, and it also attempts to evaluate the use of long term gonadectomised animals as models for testing negative feedback.

5.2 The effect of gonadectomy on LH secretion

5.2.a Procedure

Five adult male and five adult female marmosets were bilaterally gonadectomised under anaesthesia. Collection of blood began 3 days before the operations and continued at 3 day intervals for 18 days. An extra blood sample was taken 25 days after gonadectomy. Results from one female which died before the experiment was completed have not been included. Approximately 20 weeks after gonadectomy animals were bled at 0.5h intervals for 4h.

5.2.b Results

Mean LH concentrations (± S.E.M.) before and after gonadectomy are shown in Figure 5.1. No significant differences between the sexes in the rates of increase in circulating LH levels after gonadectomy could be ascertained ($p > 0.2$, student's t-test, at each time). The rates of increase in the concentration of LH and the maximum concentrations attained after gonadectomy were similar in all animals. In both males and females, a clear increase ($p < 0.01$, paired t-test) in the level of LH in the plasma was observed by day 3 after gonadectomy, and in most animals LH concentrations reached a plateau by days 9-12. Mean (± S.E.M.) plasma LH concentrations 10 weeks after gonadectomy (measured in samples taken just before the insertion of oestradiol-17β implants, Fig. 5.3) were not significantly higher than those observed after 15 days ($p > 0.2$, paired t-test).

Plasma LH concentrations in marmosets bled at 0.5h intervals 20 weeks after gonadectomy are shown in Figure 5.2. In each animal, secretion of LH was episodic with as many as four peaks occurring within the 4h period. The frequency of the pulsatile discharges of LH appears to be circhoral in four of the animals. In the remainder however, the periodicity of LH release cannot be determined from the sampling frequency used.

5.3 The effect of oestradiol-17β implants on LH secretion in gonadectomised marmosets.

5.3.a Procedure

Silastic implants containing crystalline oestradiol-17β were introduced into all animals approximately 10 wks after gonadectomy. Blood samples, taken 3 days before and 0, 1, 4, 8, 12, 22,

Figure 5.1

The effect of gonadectomy on plasma LH concentrations in male and female marmosets

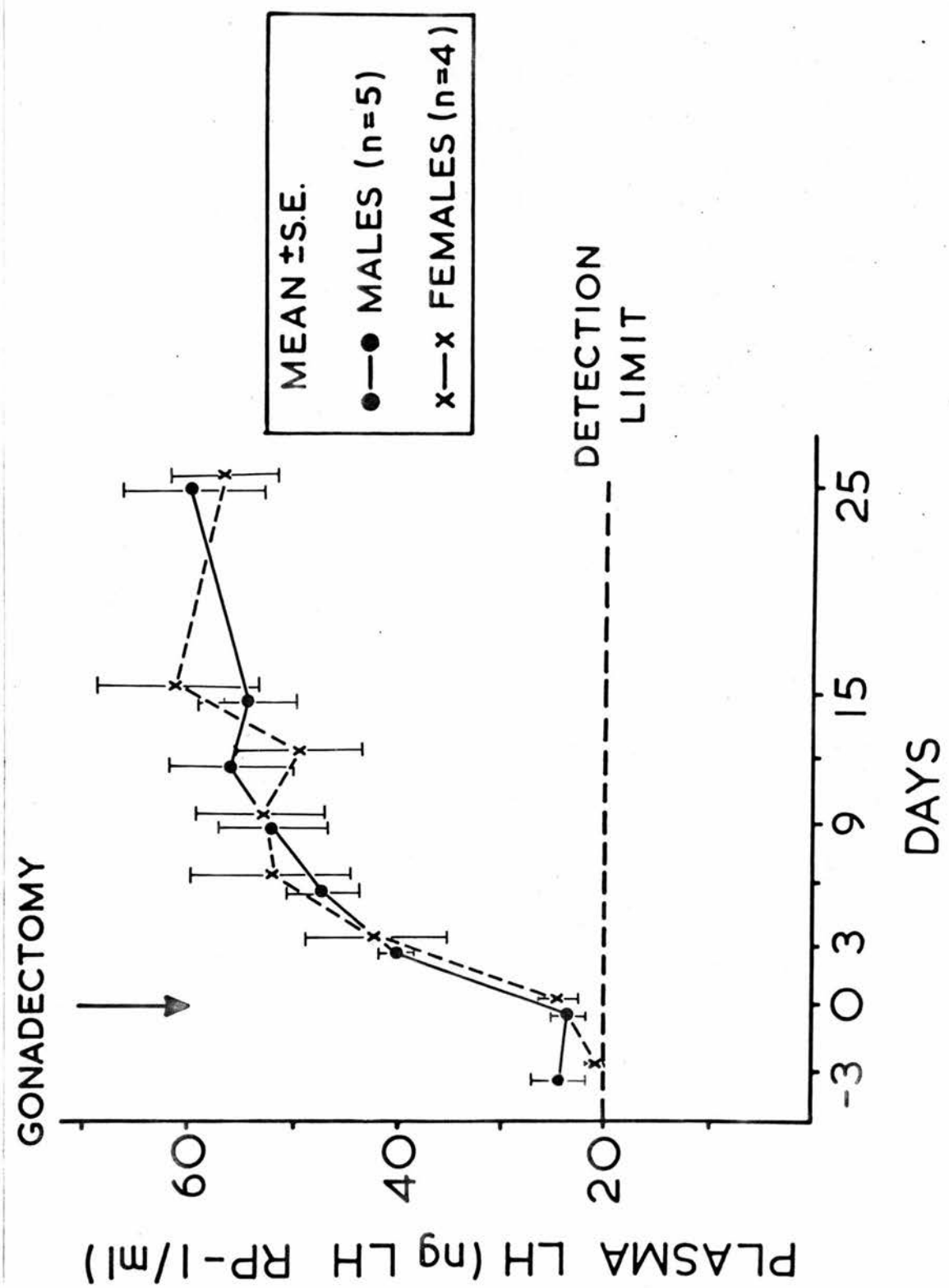
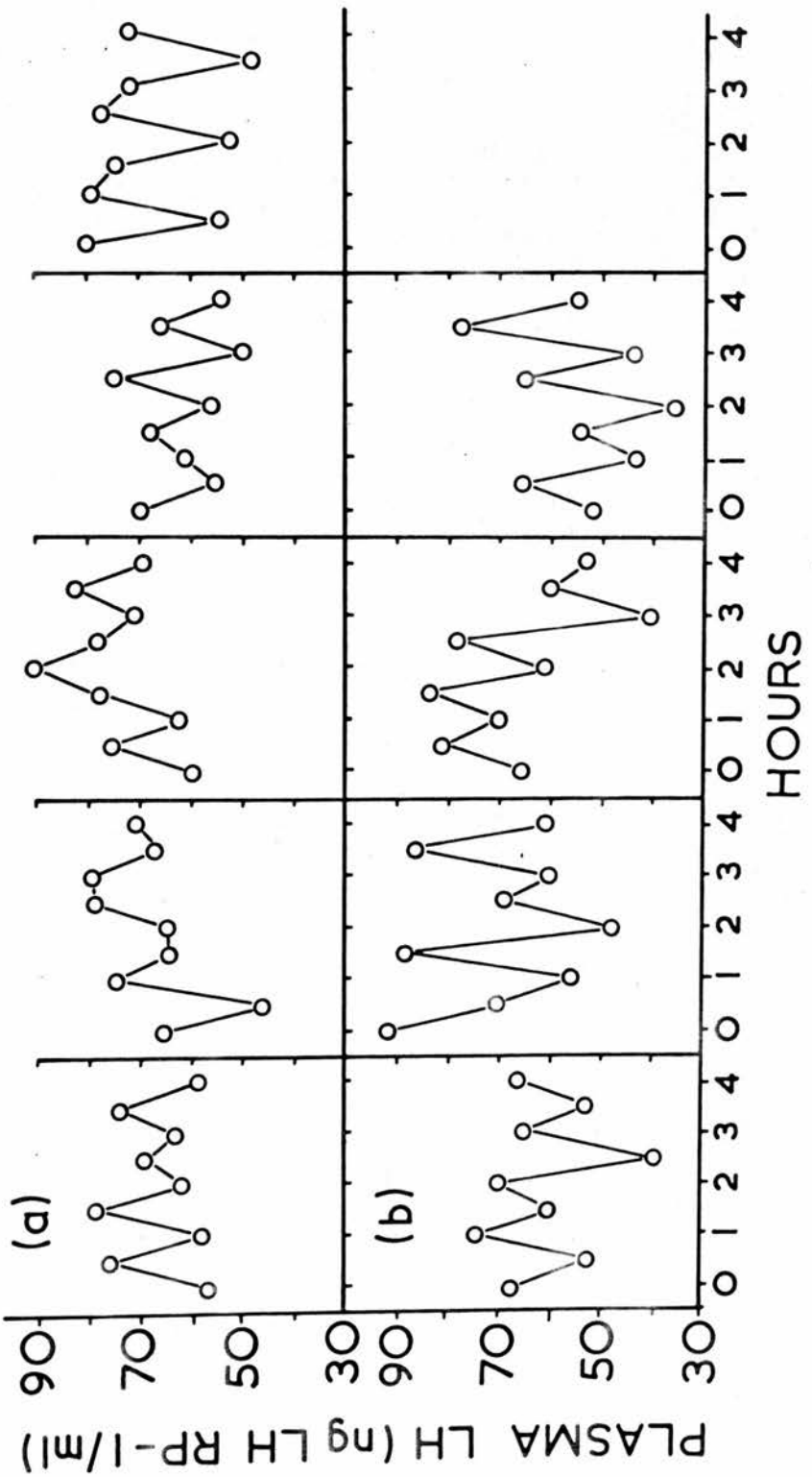


Figure 5.2

Plasma LH concentrations in individual male (a) and female (b) long term gonadectomised marmosets



26 and 36 days after the insertion of the implants were assayed for LH and oestradiol-17β . A release rate of 10-20ug oestradiol / day by the implants was estimated from prior incubation in vitro.

5.3.b Results

The effect of oestradiol-17β on plasma LH concentrations in gonadectomised marmosets is shown in Figure 5.3. 24h after the implants were introduced plasma LH concentrations were significantly elevated ($p < 0.01$ compared with pre-implant levels; paired t-test) in both males and females. This stimulatory effect of oestradiol on LH secretion was followed by a marked inhibitory action. Eight days after the implants were introduced plasma LH concentrations were significantly depressed and LH levels were undetectable for the rest of the study. From the sampling frequency used it was not possible to detect any difference between the LH response in males and females (the difference between plasma LH levels in male and female marmosets on day 4 was not significant; $p > 0.05$ student's t-test). The levels of oestradiol-17β produced by the implants are shown in Figure 5.3. There was an initial peak in the concentration of plasma oestradiol on day 1, coincident with the LH peak, followed by a decline to fairly constant levels with mean ($\pm$ S.E.M.) values ranging from 0.53 ($\pm$ 0.05) to 0.61 ($\pm$ 0.05) ng/ml.

5.4 The effects of progesterone, testosterone and dihydrotestosterone on LH secretion in long term gonadectomised marmosets

5.4.a Procedure

Male and female marmosets gonadectomised at least 3 months previously received the following treatment:

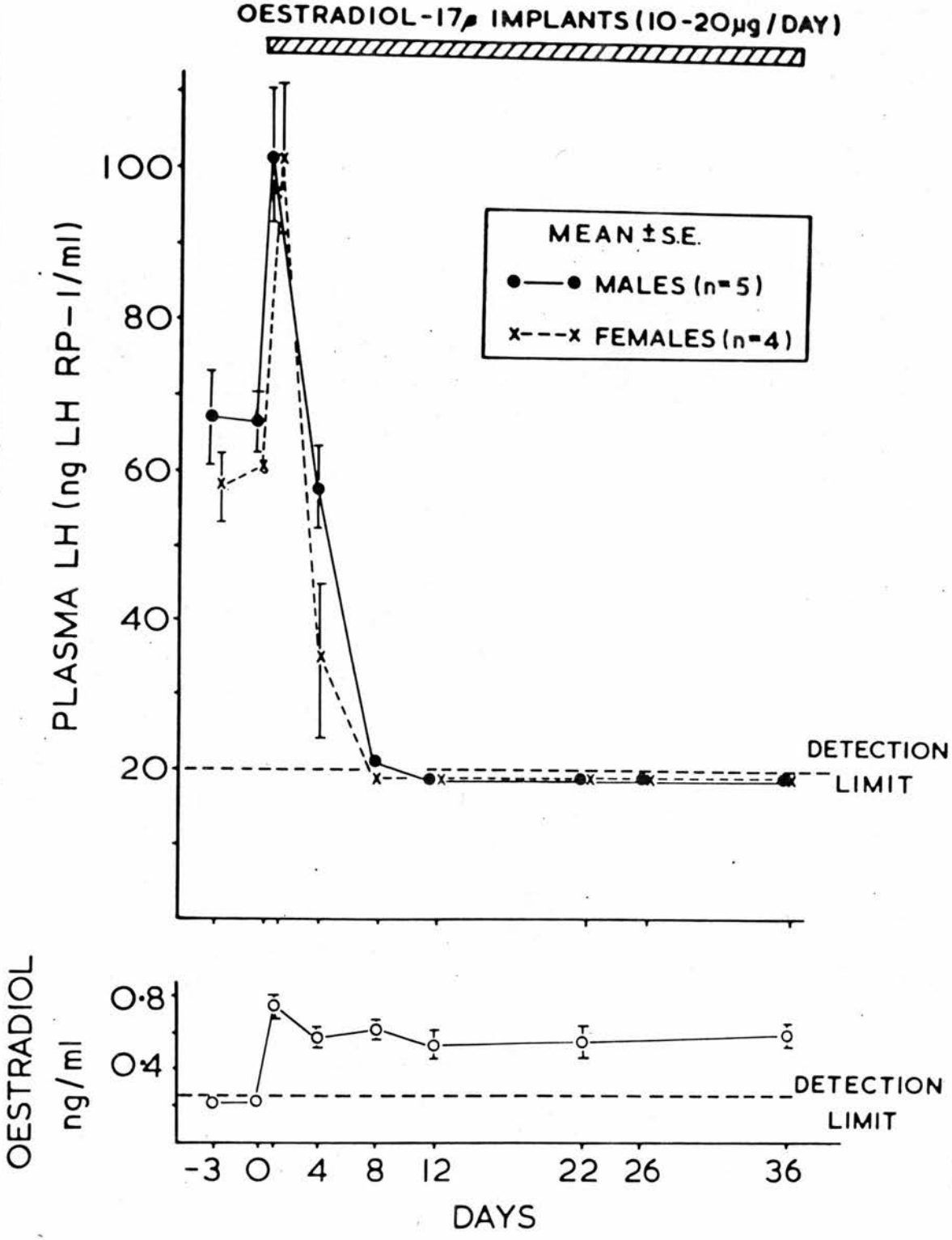


Figure 5.3

Plasma LH concentrations in male and female long term gonadectomised marmosets before and after the introduction of oestradiol-17β implants. Mean (± S.E.M.) (n=9) plasma oestradiol-17β concentrations are also shown

Three groups of animals were given daily sub-cutaneous injections of 1.0mg progesterone (P) (group 1; 5 males, 5 females), 0.5mg testosterone (T) (group 2; 4 males, 4 females) or 0.5mg dihydrotestosterone (DHT) (group 3; 4 males, 4 females) in 0.1ml arachis oil for 3 days. Blood samples, taken immediately before each injection and 24h after the last injection, were assayed for LH, and either P (group 1) or T (group 2).

In addition, two groups of marmosets, each comprising 3 males and 3 females, were given sub-cutaneous injections of 0.5mg T or 0.5mg DHT in 0.1ml arachis oil every 8h for 32h. Blood samples were taken every 8h for 40h, and assayed for LH and T. Control animals received either 0.1ml arachis oil (3 males, 3 females) or no treatment (3 males, 3 females) and were bled after 0, 8, 24, 36 and 48h.

5.4.b Results

Injections of arachis oil had no effect on LH secretion. LH concentrations in oil-treated and non-treated animals have therefore been combined to produce mean values (Table 5.1). Since LH secretion in gonadectomised marmosets is episodic, considerable variation between sequential blood samples is expected. In an attempt to distinguish between suppression of LH secretion due to negative feedback and fluctuations in LH levels associated with pulsatile release, negative feedback has been defined as a reduction in LH secretion exceeding two standard deviations below mean control values. In Figures 5.4 - 5.8 the dotted area represents the limits formed by taking 2 standard deviations above and below the mean control values, and a fall in LH concentration below this area following steroid treatment will be classified as negative feedback.

ANIMAL	TIME (HOURS) 0	8	24	36	48
♂ 1*	48	74	51	52	63
2*	71	55	64	73	73
3*	68	60	68	49	50
Mean (n=3)	62.3	63.0	61.0	58.0	62.0
S.D.	12.5	9.8	8.9	13.0	11.5
♂ 4	83	72	70	64	68
5	71	66	67	69	72
6	61	68	46	50	51
Mean (n=3)	71.7	68.7	61.0	61.0	63.7
S.D.	11.0	3.0	13.1	9.8	11.1
Mean (n=6)	67.0	65.8	61.0	59.5	62.8
S.D.	11.7	7.2	10.0	10.5	10.2
-2SD - +2SD	43.6-90.4	51.4-80.2	41.0-81.0	38.5-80.5	42.4-83.2
♀ 1*	53	69	51	61	68
2*	76	62	48	59	56
3*	56	48	73	72	81
Mean (n=3)	61.7	59.7	57.3	64.0	68.3
S.D.	12.5	10.7	13.7	7.0	7.2
♀ 4	62	46	56	80	69
5	55	59	67	62	70
6	81	66	64	59	51
Mean (n=3)	66.0	57.0	62.3	67.0	63.3
S.D.	13.5	10.1	5.6	11.4	10.7
Mean (n=6)	63.8	58.3	59.8	65.5	65.8
S.D.	11.8	9.4	9.7	8.6	10.7
-2SD - +2SD	40.2-87.4	39.5-77.1	40.4-79.2	48.3-82.7	44.4-87.2

Table 5.1

Individual LH concentrations in control gonadectomised male and female marmosets, receiving arachis oil at time 0 (*), or no treatment. Means, S.D.'s and the limits formed by taking 2 S.D.'s above and below mean values are also given

The effects of daily injections of P, T and DHT to long term gonadectomised marmosets are shown in Figures 5.4, 5.5 and 5.6 respectively. From the individual results it can be seen that although there was considerable fluctuation in LH concentrations, none of the steroids tested had a negative feedback effect on LH secretion over the first 2 days. Although control samples were taken only over the first two days, this was sufficient to show the extent of the fluctuation in LH concentrations due to episodic or diurnal variation, and from the individual LH levels shown in Figures 5.4 - 5.6, it would be reasonable to conclude that there was no negative feedback over the entire 3 day period. Mean LH concentrations after the initiation of steroid treatment were not significantly different from pre-injection values ($p > 0.05$, paired t-test), and comparisons between LH levels at each of the times during the experiments in males and females also showed no significant differences ($p > 0.05$, student's t-test). Circulating levels of progesterone and testosterone achieved by the injections are shown in Figures 5.4 and 5.5. Mean plasma concentrations of P and T 24h after each injection were between 60 and 90 ng/ml and 5 and 10 ng/ml respectively. Circulating steroid levels were similar in males and females.

Administration of T and DHT every 8h for 32h also had no apparent effect on LH secretion in long term gonadectomised marmosets (Figs. 5.7 and 5.8), and individual results show that there was no negative feedback in any of the animals tested. There were no significant differences either between LH levels in males and females ($p > 0.05$ student's t-test), or between LH concentrations after the initiation of steroid treatment and pre-injection values ($p > 0.05$, paired t-test). Testosterone concentrations 8h after each injection (approximately 35-50 ng/ml) were similar in males and females.

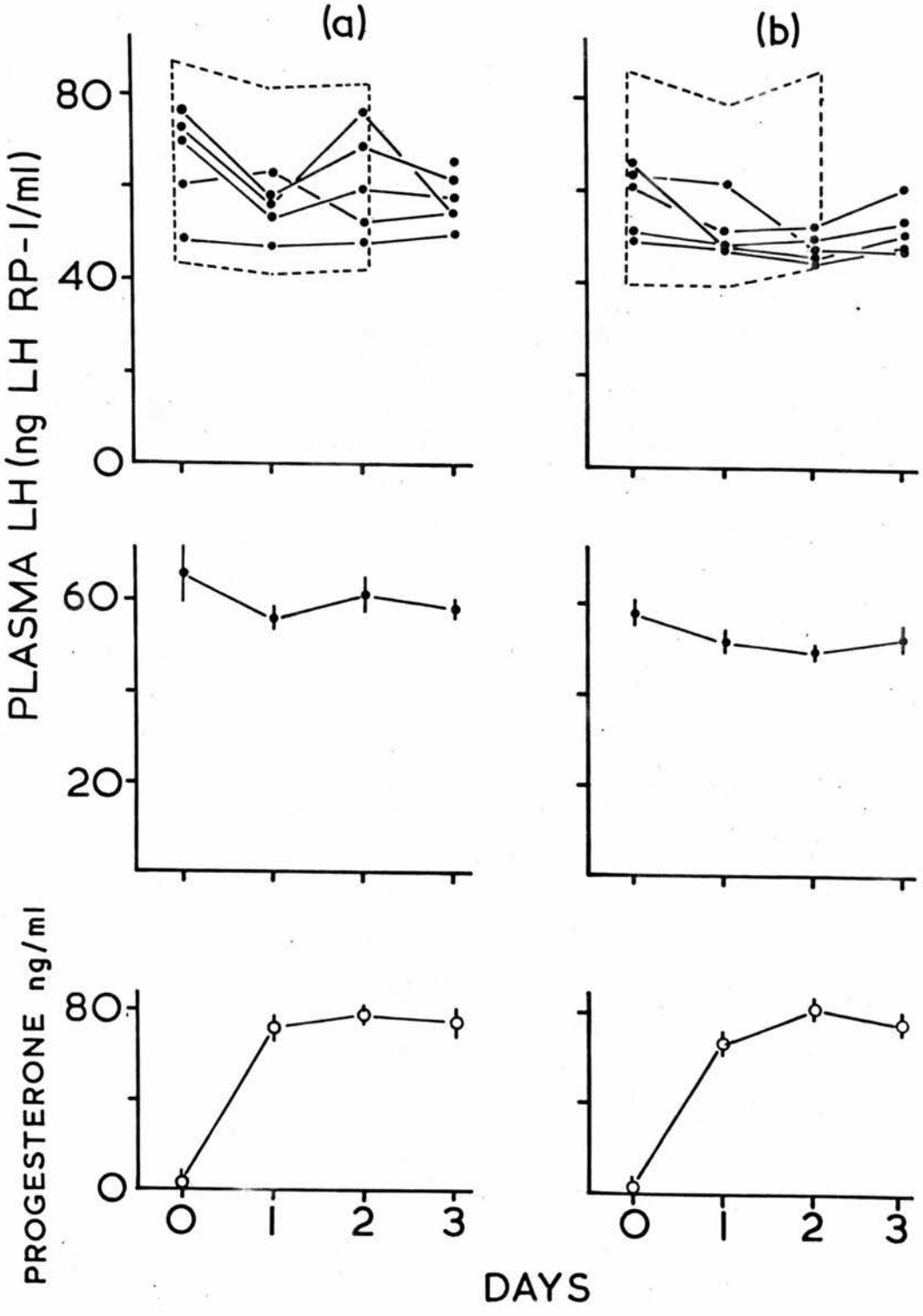


Figure 5.4

The effect of daily s.c. injections of 1.0mg P in oil on LH concentrations in long term gonadectomised males (a) and females (b)

LH concentrations are shown as individual, and Mean ($\pm$ S.E.M.) values (n=5). Progesterone levels are Mean ($\pm$ S.E.M.), n = 5.

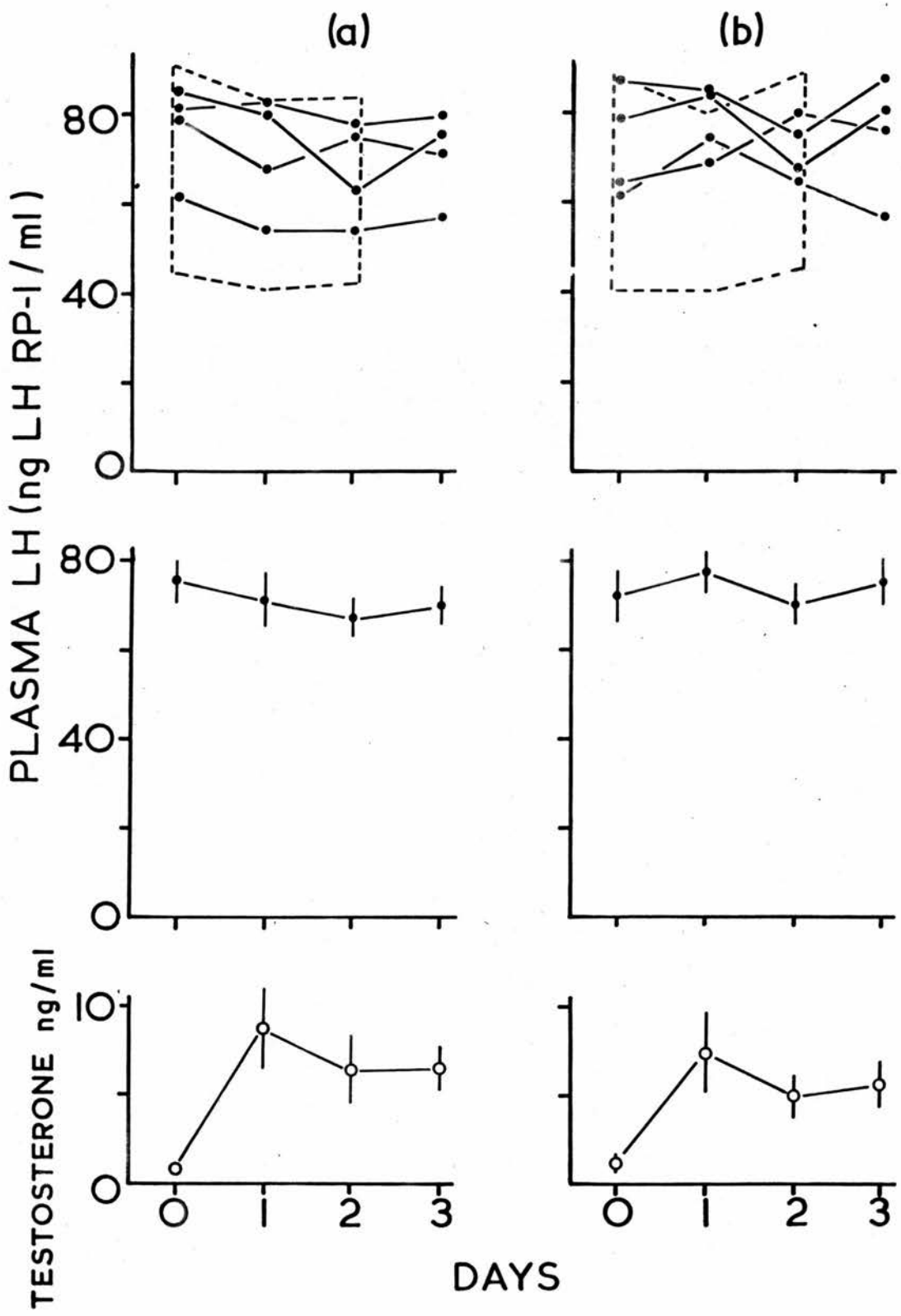


Figure 5.5

The effect of daily s.c. injections of 0.5mg T in oil on LH concentrations in long term gonadectomised males (a) and females (b)

LH concentrations are shown as individual, and Mean (± S.E.M.) values (n=4). Testosterone levels are Mean (± S.E.M.), n = 4.

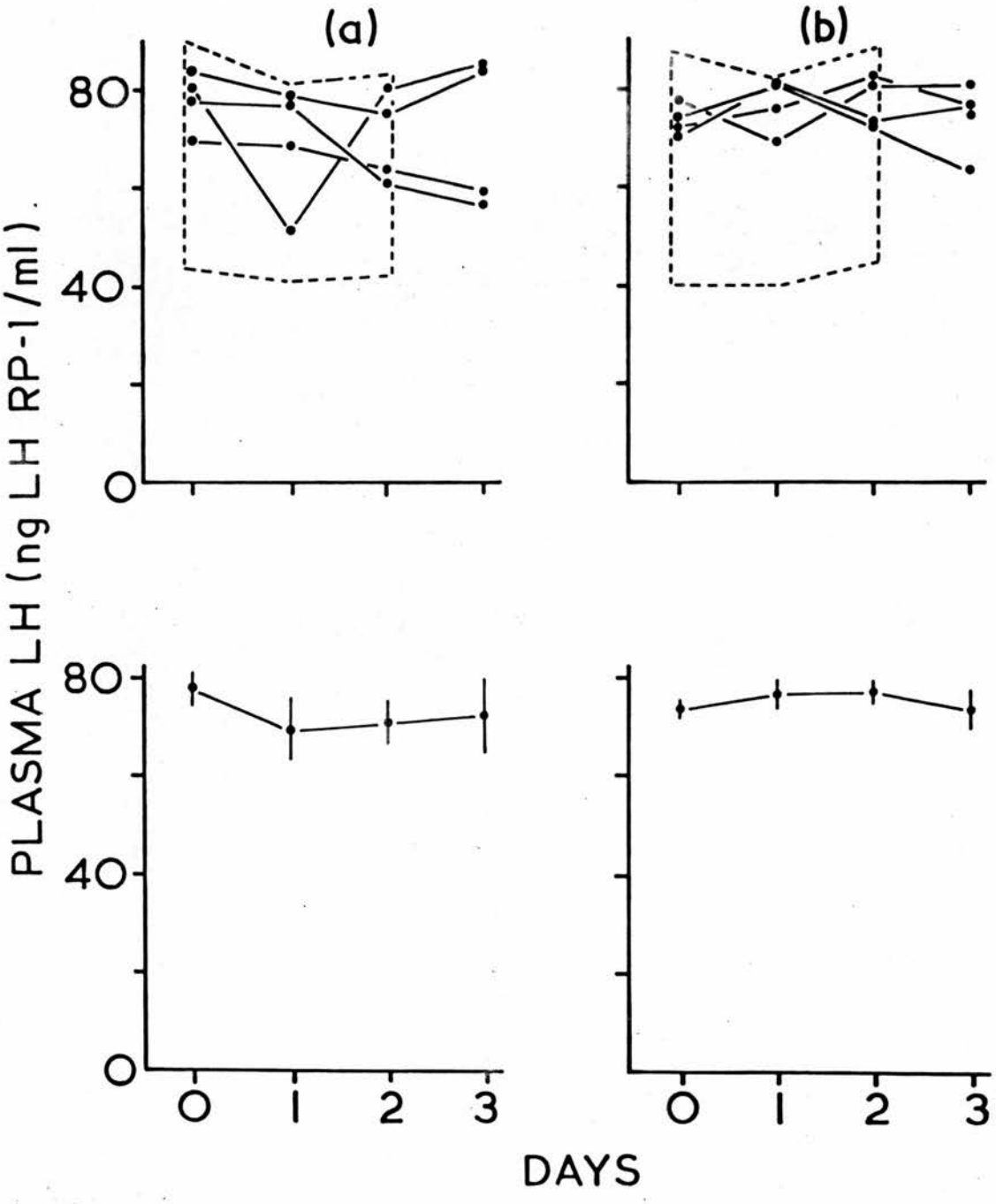


Figure 5.6

The effect of daily s.c. injections of 0.5mg DHT in oil on LH concentrations in long term gonadectomised males (a) and females (b)

LH concentrations are shown as individual, and Mean ($\pm$ S.E.M.) values (n=4).

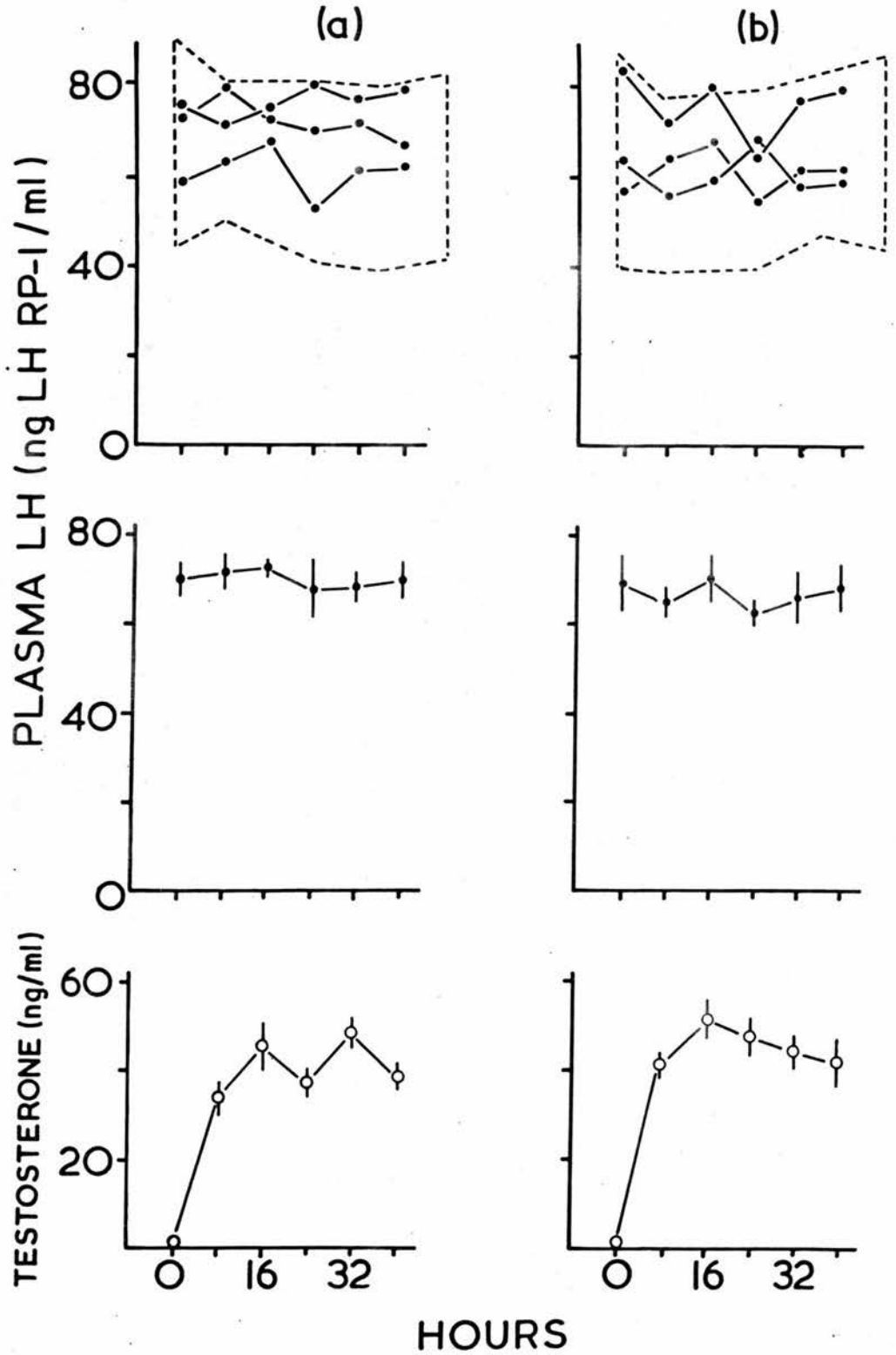


Figure 5.7

The effect of 8h s.c. injections of 0.5mg T in oil on LH concentrations in long term gonadectomised males (a) and females (b)

LH concentrations are shown as individual, and Mean (± S.E.M.) values (n=3). Testosterone levels are Mean (± S.E.M.), n=3.

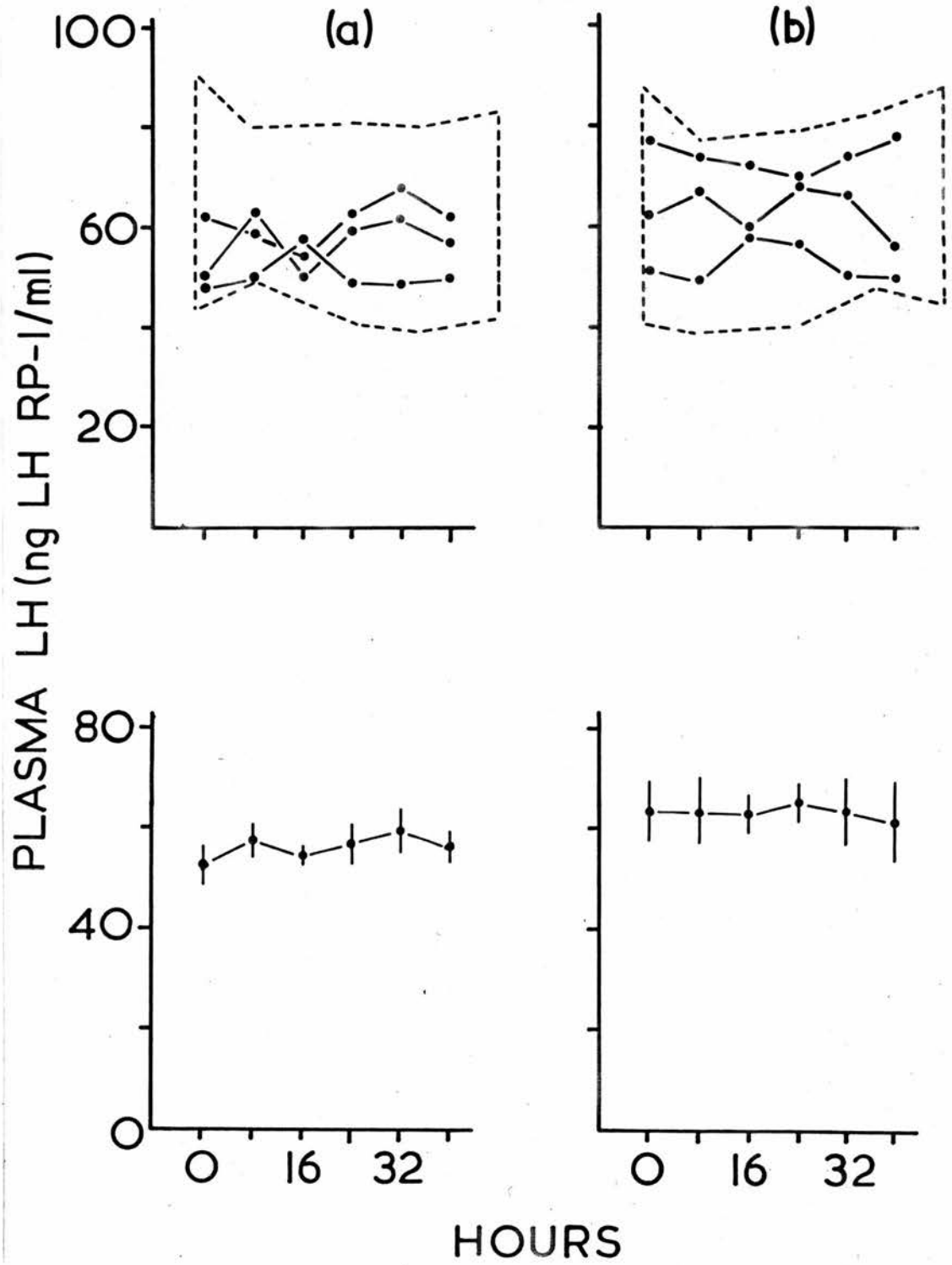


Figure 5.8

The effect of 8h s.c. injections of 0.5mg DHT in oil on LH concentrations in long term gonadectomised males (a) and females (b)

LH concentrations are shown as individual, and Mean (± S.E.M.) values (n=3).

5.5 The effects of progesterone, testosterone and dihydrotestosterone on LH secretion in short term gonadectomised marmosets

5.5.a Procedure

Five adult male and 5 adult female marmosets were bilaterally gonadectomised and then given daily injections of 1.0 mg P in 0.1ml arachis oil for 8 days, beginning on the day of gonadectomy. Blood samples were taken once daily for 8 days and were assayed for LH and P. Male and female marmosets which did not receive steroid treatment following gonadectomy (Fig. 5.1) were used as controls.

Two groups of gonadectomised marmosets each comprising 5 males and 5 females received oestradiol-17β implants (releasing 10-20 ug oestradiol-17β /day) 3 weeks before the start of the experiment. Physiological castration was staged by removing the implants, and daily injections of 0.5 mg T or 0.5 mg DHT in 0.1 ml arachis oil were administered for 8 days, beginning on the day when the implants were removed. Daily blood samples were taken throughout the 8 day period and were assayed for LH and T. Gonadectomised controls (3 males and 3 females) received similar treatment except that they were not given steroid injections after the removal of the oestradiol-17β implants.

5.5.b Results

Daily injections of progesterone inhibited the post castration rise in LH levels in male and female marmosets (Fig. 5.9). Mean LH levels 6 days after gonadectomy were significantly lower in animals receiving progesterone than in the controls ($p < 0.05$, males: $p < 0.01$ females, student's t-test), and there was no significant difference between the LH response in males and females ($p > 0.05$ student's t-test). However, LH levels in females before gonadectomy

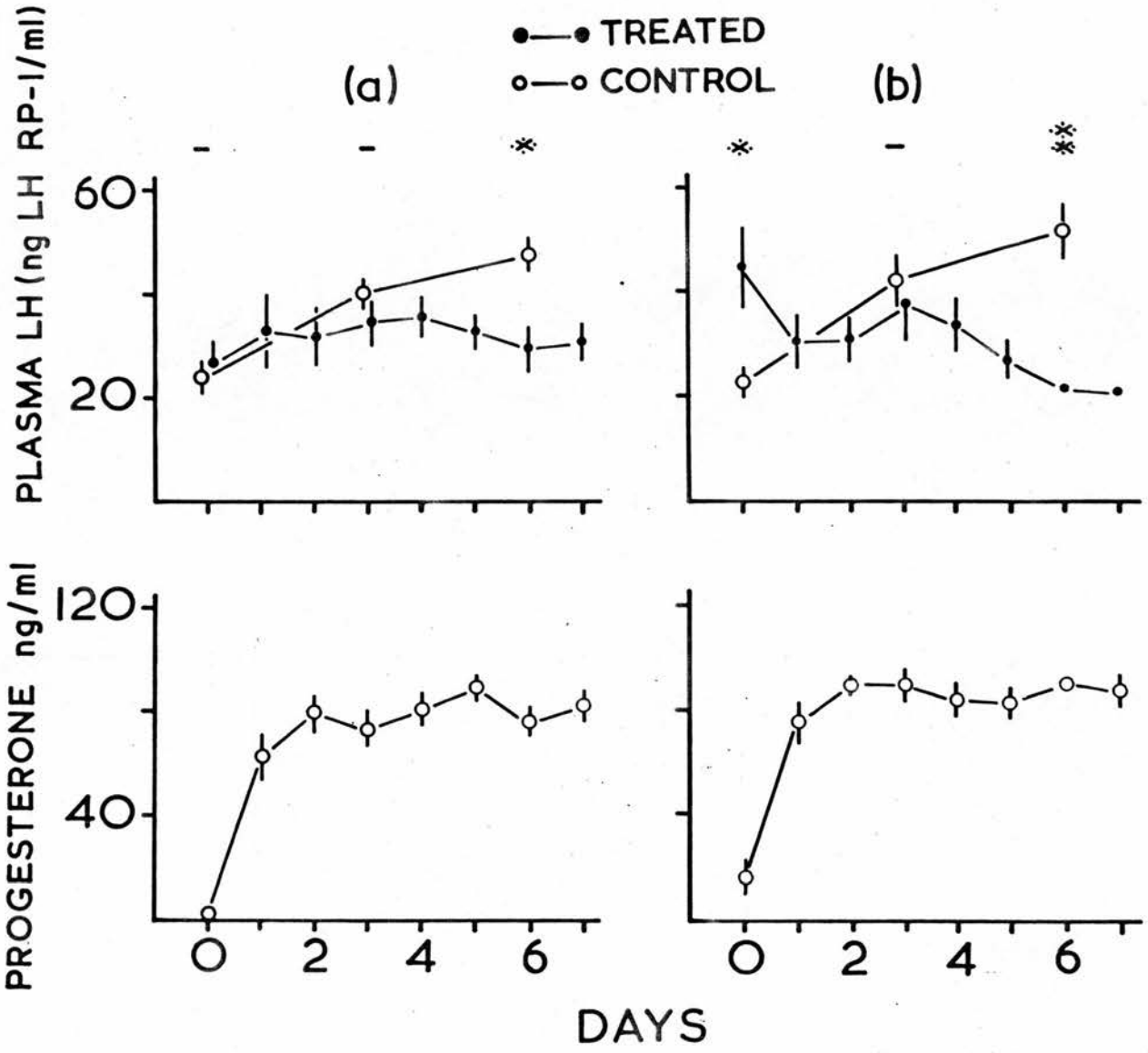


Figure 5.9

The effect of daily s.c. injections of 1.0mg P in oil on LH concentrations in short term gonadectomised males (a) and females (b)

Gonadectomy was performed on day 0.

Progesterone and LH values are Mean ± S.E.M.; treated animals n=5, controls n=5 (males), n=4 (females).

-, N.S.; *, $p < 0.05$; ** $p < 0.01$, compared with controls.

were significantly higher than control values ($p<0.05$, student's t-test). Mean plasma progesterone concentrations 24h after each injection (approximately 60-90 ng/ml) were similar to those observed in the experiment on long term gonadectomised animals.

Oestradiol-17β implants effectively suppressed LH secretion, and although circulating levels of oestradiol were not measured, implants of the same size when used in a previous experiment, produced mean ($\pm$ S.E.M.) oestradiol concentrations ranging from 0.53 ($\pm$ 0.05) to 0.61 ($\pm$ 0.05) ng/ml (Fig. 5.3). The pattern of LH secretion after the removal of oestradiol implants (with no further steroid treatment) did not differ significantly from that observed after gonadectomy over the period studied ($p>0.05$ after 3 and 6 days, student's t-test).

Daily injections of testosterone inhibited the rise of LH following the removal of the oestradiol implants in females, but not in males (Fig. 5.10). Whereas mean LH levels in males receiving testosterone were not significantly different compared with controls ($p>0.2$ student's t-test), there was a significant difference between testosterone treated females and controls on days 2, 4 ($p<0.05$), 6 and 7 ($p<0.01$, student's t-test). Although there was no apparent difference between circulating testosterone levels in males and females, LH levels were significantly higher in males than in females from day 2 onwards ($p<0.05$, student's t-test).

In contrast to the effects of T, DHT prevented the rise in LH concentrations following the removal of oestradiol implants in males, but not in females (5.11). There was a significant difference in mean LH concentrations between DHT treated males and controls by day 3, and between males and females by day 5 ($p<0.01$, student's t-test).

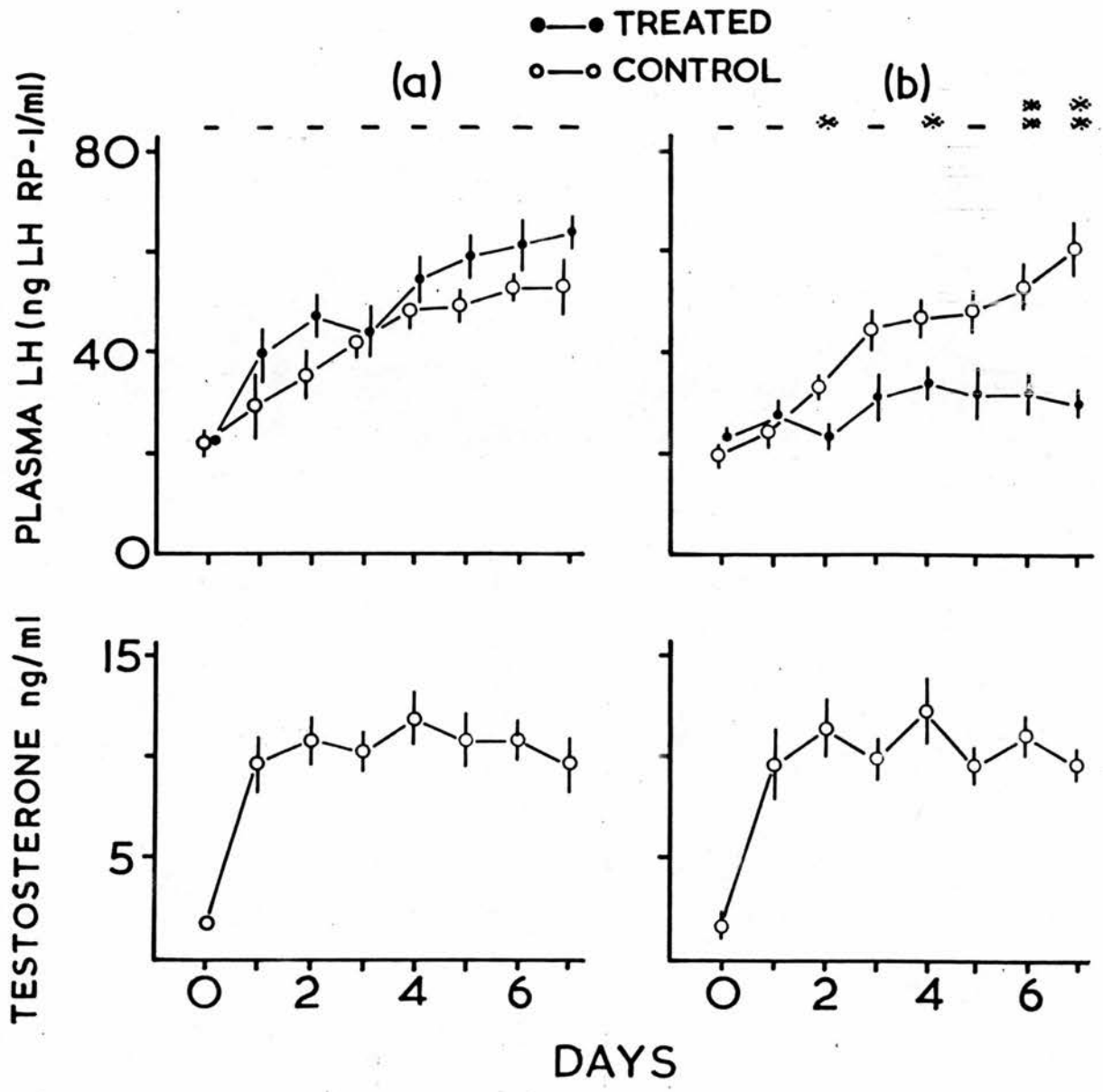


Figure 5.10

The effect of daily s.c. injections of 0.5mg T in oil on LH concentrations in short term gonadectomised males (a) and females (b)

Oestradiol implants were removed on day 0.

Testosterone and LH values are Mean ± S.E.M.; treated animals n=5, controls n=3.

-, N.S.; * $p<0.05$; ** $p<0.01$, compared with controls.

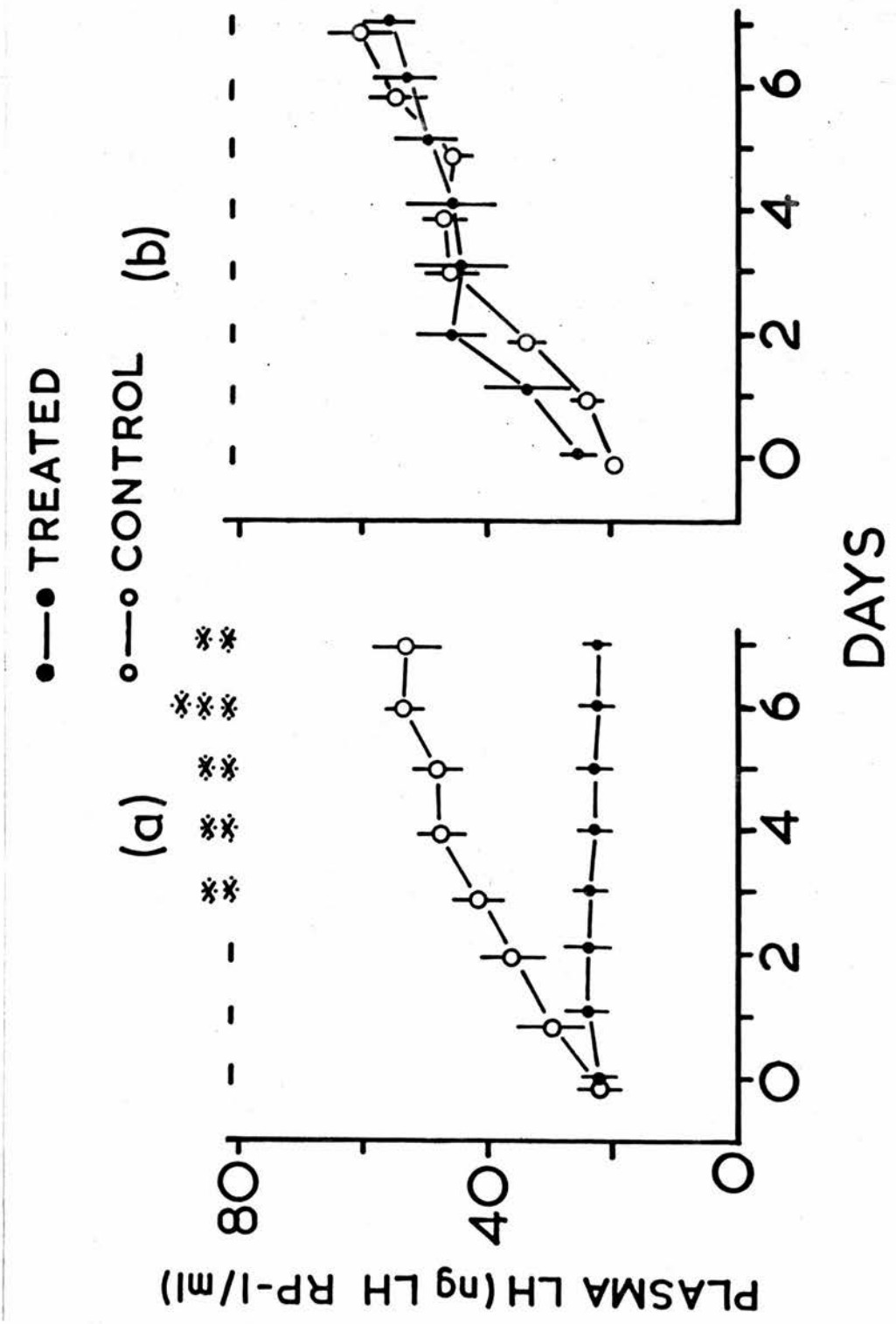
Figure 5.11

The effect of daily s.c. injections of 0.5 mg DHT in oil on LH concentrations in short term gonadectomised males (a) and females (b)

Oestradiol implants were removed on day 0.

LH values are Mean ± S.E.M.; treated animals n=5, controls n=3.

-, N.S.; ** $p < 0.01$; *** $p < 0.001$, compared with controls.



5.6 Discussion

Secretion of LH by the pituitary gland increases markedly after gonadectomy in male and female marmosets. The greatest increment in the concentration of LH occurred during the first 3 days, after which LH levels continued to rise until a plateau was reached after about 15 days. The initial increase in plasma levels of LH almost certainly occurred earlier than day 3, but since the first sample was not collected until this time there are no data to show this. However, Reeves et al (1972) observed that plasma LH levels increased within 12h of castration in the ram, and Goldman and Porter (1970) reported that female hamsters ovariectomised during dioestrus have markedly increased levels of LH within 3.5h of gonadectomy. In male and female rhesus monkeys (Atkinson et al, 1970), women (Yen and Tsai, 1971b) and men (Seyler and Reichlin, 1974b) increases in plasma LH levels occur 1-2 days after gonadectomy. In women it has been shown that during the first week after gonadectomy a significantly greater rise in LH is observed in those subjects ovariectomised during the follicular phase than in those ovariectomised during the luteal phase of the menstrual cycle (Yen and Tsai, 1971b). In the present study, the exact stage of the cycle at which ovariectomy was performed was not known and it is therefore not possible to determine whether the rate of increase in LH levels in the marmoset varies according to the stage of the cycle at which ovariectomy is performed. In agreement with work on the rhesus monkey (Atkinson et al, 1970) there does not appear to be a sex difference in the rate of increase in LH levels after gonadectomy in the marmoset.

Blood samples collected at 0.5h intervals revealed an episodic release of LH in gonadectomised marmosets. Fluctuations in plasma LH levels have been observed in a number of other species (see page 8 for references) although the frequency of the discharges appears to vary between species. In the present study the rhythm of LH discharge was circhoral (as in the rhesus monkey, Dierschke et al, 1970) in 4 animals, but appeared to be irregular in the remainder. Reeves et al (1972) and Dierschke et al (1970) showed in the ewe and rhesus monkey respectively that regular periods of LH discharge could be detected only when the sampling frequency was of the order of every 10-20 min. Hourly blood samples were initially taken in these species (Atkinson et al, 1970; Reeves et al, 1972) and in both cases LH discharges appeared completely random. If a greater sampling frequency had been used in the present study a regular (circhoral) period of LH discharge may have been observed in more of the animals.

Implantation of silastic capsules containing crystalline oestradiol-17β inhibited LH secretion in male and female gonadectomised marmosets. This demonstration of a negative feedback effect of oestradiol confirms earlier reports (Legan, Gay and Midgley, 1973; Karsch et al, 1973a)that this steroid is capable, on its own, of restoring the negative feedback loop between the gonad and the hypothalamo-hypophysial axis which is interrupted by gonadectomy. The rate of oestradiol released by the implants, estimated to be between 10 and 20 ug/day, produced plasma oestradiol concentrations (after the peak on day 1) similar to those found in the late follicular phase of the ovarian cycle in the intact female marmoset (Hearn and Lunn, 1975). Coincident with the peak in oestradiol-17β levels on day 1 was a marked increase in LH secretion in all animals,

indicating that oestradiol-17β is capable of exerting both negative and positive feedback actions. Furthermore the results obtained here suggest that castrated male (as well as castrated female) marmosets are sensitive to a positive feedback action of oestrogen, and will release LH when stimulated with this hormone.

Detailed analysis of the effects of oestradiol on LH secretion, particularly work on the rhesus monkey (Karsch et al, 1973a), has shown that the negative feedback control system governing LH secretion is remarkably sensitive to small changes in plasma oestradiol concentrations, and that irrespective of duration, negative feedback can be induced by considerably lower circulating levels of oestradiol than positive feedback. Thus, if lower levels of oestrogen had been used in the present study it is probable that only the negative feedback response would have been observed, although the suppression of LH levels may not have been as marked.

In contrast to the inhibitory efficacy of oestradiol-17β, progesterone, testosterone and dihydrotestosterone, under the experimental conditions described, had no apparent negative feedback effect on LH secretion in long term gonadectomised marmosets. The definition of negative feedback used in this study may be considered to be severe, but it is not unreasonable in view of the considerable fluctuations in LH levels associated with pulsatile secretion. Furthermore, there was no decrease in mean LH concentrations with any of the steroids administered during the test period. The possibility that negative feedback did occur but was not detected is therefore unlikely, but should not be completely excluded.

The levels of progesterone and testosterone shown in Figures 5.4 and 5.5 represent the hormone concentrations 24h after the

injections, and it must therefore be assumed that the maximum levels achieved were considerably higher than is shown. Since progesterone concentrations 24h after the injection were approaching the upper limit of mid luteal phase levels, the maximum concentrations achieved would almost certainly have been supraphysiological. Testosterone concentrations 24h after each injection however, were at the lower end of the normal male range, and may not have been elevated for long enough to suppress LH. However, testosterone concentrations when maintained in the region of 40 ng/ml (by 8h injections) were also unable to inhibit LH secretion. Dihydrotestosterone concentrations were not measured. However, since the treatment with dihydrotestosterone was identical to that of testosterone, the effects of the two hormones may be compared, albeit with caution.

There is a strong possibility that the injections of steroids were not continued for long enough to inhibit LH secretion. However studies on long term gonadectomised rhesus monkeys have also failed to demonstrate a negative feedback effect of progesterone either with physiological doses maintained for several months (Karsch et al, 1973a) or with pharmacological doses maintained for 2 weeks (Yamaji et al, 1972). Similarly, physiological doses of testosterone when maintained for 3 weeks were unable to suppress LH secretion in long term gonadectomised male rhesus monkeys (Resko et al, 1976). Nevertheless, it should be emphasised that a more complete examination using several doses and duration of treatment is necessary before any firm conclusions regarding the negative feedback action of any of the steroids tested in long term gonadectomised marmosets can be drawn.

In contrast to the apparent ineffectiveness of progesterone, testosterone and dihydrotestosterone to suppress LH secretion in

long term gonadectomised marmosets, all three steroids had a negative feedback action in short term gonadectomised animals. The results for progesterone agree with the observations of Karsch et al (1976) which showed that treatment with progesterone immediately following gonadectomy prevented the typical post castration rise in circulating LH levels in the ewe. They are however, in contrast to the observations in the long term ovariectomised rhesus monkey (Yamaji et al, 1972; Karsch et al, 1973a), from which it was concluded that progesterone alone is physiologically inert in the control of tonic LH secretion in primates. It is unlikely that these contrasting observations are due simply to methodological or species differences, but they may be related to the interval between gonadectomy and the initiation of progesterone treatment. In this regard, the efficacy of another ovarian steroid, oestradiol-17β , to inhibit LH secretion appears to decrease with time after ovariectomy in sheep (Brown, Cumming, Goding and Hearnshaw, 1972) as well as in rats (Legan and Karsch, 1975).

Although there is a clear difference between the feedback effects of exogenous progesterone in long term and short term gonadectomised marmosets, the results do not necessarily imply that progesterone normally exerts a negative feedback action in intact animals. Maximum progesterone concentrations were almost certainly supraphysiological, and the possibility that the exogenous progesterone was converted to another steroid which was active in suppressing LH secretion cannot be excluded. Nevertheless, the results indicate that under the experimental conditions described, progesterone treatment can exert a negative feedback effect on LH secretion in the marmoset. They also suggest that it may be misleading to interpret the negative

feedback properties of progesterone in long term gonadectomised animals as representing the effects of the hormone in other physiological conditions.

The feedback effects of testosterone and dihydrotestosterone also appear to be different in long term and short term gonadectomised marmosets. However, conclusions based on the present results must be tempered by the fact that, due to a shortage of animals available for experimentation, castration was physiologically staged by removing the suppressive effects of oestradiol-17β implants, and that actual gonadectomy was not performed. Although the implants chronically suppressed LH secretion, the hormonal environment under these conditions is not the same as in intact animals, and the levels of circulating oestradiol produced by the implants (assumed to be in the region of 0.5 - 0.6 ng/ml) would almost certainly be well in excess of the levels of endogenous oestradiol in intact male marmosets. As oestradiol has been shown to increase the number of androgen receptors in the chick oviduct (Harrison and Toft, 1973), the possibility that oestrogens affect the sensitivity to androgens in the marmoset cannot be excluded.

Nevertheless, the results show that under the experimental conditions testosterone and dihydrotestosterone were able to exert a negative feedback effect on LH secretion. The reason why testosterone was effective in only the females and dihydrotestosterone in only the males is not clear from the data. The feedback effect of testosterone may be due to the hormone per se, or it may be due to some other steroid to which testosterone is converted. Since testosterone can be converted to oestradiol-17β, either in the peripheral circulation (Longcope, Kato and Horton, 1969) or in the brain (Ryan, Naftolin, Reddy, Flores and Petro, 1972; Naftolin, Ryan, Davies, Reddy, Flores,

Petro, White, Kuhn, Takaoka and Wolin, 1975), oestradiol-17β may be responsible for mediating the negative feedback effects of testosterone. In this regard, it has been shown that gonadectomised male rhesus monkeys are less sensitive to the negative feedback action of low doses of oestradiol than gonadectomised females (Steiner et al, 1976). Whether or not the effects of testosterone in the present study were mediated through oestrogen, larger doses and more prolonged treatment may have prevented the rise in LH levels in males as well as in females. Although physiological doses of testosterone alone do not appear to exert a negative feedback effect on LH secretion in either long term or short term gonadectomised male rhesus monkeys (Resko et al, 1976; 1977), testosterone has been shown to synergise with sub-threshold doses of oestradiol (Resko et al, 1977). In accord with the present results, Resko et al (1977) found that the feedback efficacy of testosterone (albeit in combination with oestradiol) was related to the interval between gonadectomy and the initiation of steroid treatment, and that LH secretion was inhibited only in short term gonadectomised animals.

Dihydrotestosterone is a substance with a reduced A-ring which is not convertible to oestradiol-17β, or other known oestrogens (Ito and Horton, 1971). It is therefore reasonable to assume that the feedback effects of dihydrotestosterone in the marmoset are due to a direct action of androgens. The present results in short term gonadectomised male marmosets confirm earlier reports in intact men (Stewart-Bentley et al, 1973) and in castrated male rats (Swerdloff, Walsh and Odell, 1972), that dihydrotestosterone is capable of suppressing LH secretion, and that conversion of androgens to oestrogens

may not necessarily be required to inhibit LH secretion. The feedback properties of dihydrotestosterone in females have not been previously reported.

5.7 Chapter summary

1. Bilateral gonadectomy in the marmoset results in an increase in circulating LH concentrations indicating that LH secretion is normally suppressed by the action of gonadal steroids.

2. The elevated LH concentrations in the "open-loop" situation are the result of pulsatile discharges of the hormone which in some of the animals appear to occur approximately once an hour.

3. Chronic treatment of long term gonadectomised animals with late follicular phase (approximately 500pg/ml) levels of oestradiol-17β (achieved with implants) inhibits LH secretion in males and females, although this suppressive effect was preceeded in all animals by a surge of LH 24h after insertion of the implants.

4. Under the experimental conditions described, P, T and DHT were able to prevent the post "castration" rise in LH levels in some animals, but showed no apparent inhibitory effect in long term gonadectomised marmosets. These results suggest that the sensitivity of the hypothalamic-pituitary system to negative feedback may decrease as the interval from castration increases.

5. Although these results demonstrate a negative feedback action of all the steroids tested, definition of their physiological roles in the control of tonic LH secretion in the marmoset (particularly of progesterone and androgens) requires a more thorough investigation.

CHAPTER SIX

POSITIVE FEEDBACK CONTROL OF LH SECRETION

6.1 Introduction

In addition to a negative feedback mechanism described in Chapter 5, the secretion of LH is also regulated by positive feedback. Evidence to date suggests that the most important steroid with respect to positive feedback is oestradiol-17β, and it is well established that the pre-ovulatory rise in circulating oestradiol-17β represents the critical stimulus for the initiation of the midcycle surge in women (Vande Wiele et al, 1970) and rhesus monkeys (Ferin et al, 1974a), as well as in rats (Ferin et al, 1969) and sheep (Goding et al, 1969). Progesterone and androgens may also be important in regulating the preovulatory LH surge, although the way in which they influence LH secretion is not yet clear. Thus progesterone can trigger an acute surge of LH, either in oestrogen primed hypogonadal women (Odell and Swerdloff, 1968) and men (Stearns et al, 1973), or during the late follicular phase in normally cycling women (Yen et al, 1975a), but it can also effectively block the positive feedback action of oestrogen (Netter et al, 1973; Clifton et al, 1975). Similarly, testosterone can either induce a positive discharge of LH in ewes (Clarke, 1976) or inhibit LH release in response to oestrogen stimulation in the female rat (Klawon, Sorrentino and Schalch, 1971). The experiments described in this chapter were designed to determine the characteristics of oestrogen induced positive feedback in the marmoset, and to look at the ability of physiological levels of progesterone, testosterone and dihydrotestosterone to induce, or modify oestrogen induced, positive feedback.

Elevated concentrations of oestradiol (achieved with implants) induced a positive discharge of LH in gonadectomised male as well as gonadectomised female marmosets (Chapter 5). This observation is of

interest in determining whether positive feedback in response to oestrogen is a sexually dimorphic characteristic in primates, as it is in rats (Gorski, 1971) and sheep (Karsch and Foster, 1975). Although it has been suggested (e.g. Knobil, 1974) that sexual dimorphism in the LH response to oestrogen does not exist in primates, experimental evidence to support this has not been too convincing. The action of oestradiol-17β on LH release in gonadectomised and intact male marmosets has therefore been examined.

The following experiments were done:

1. The LH response to oestradiol benzoate (ODB) in gonadectomised male and female, and intact male marmosets was measured.

2. The effects of testosterone (T), dihydrotestosterone (DHT) and progesterone (P) on ODB induced LH release in gonadectomised marmosets was determined.

3. T, DHT and P were also tested for their capacity to induce LH release in gonadectomised animals.

Eighteen gonadectomised marmosets (9 males and 9 females) were used in the experiments. In the course of the study each animal was used several times. However, care was taken to ensure that the period between two successive experiments on the same animal was as long as possible (> 2 weeks). For the purposes of this study positive feedback (negative feedback) has been defined as an increase (decrease) in plasma LH concentration exceeding two standard deviations above (below) the corresponding mean control value. While lesser responses, both negative and positive, may reflect genuine feedback effects, it was felt necessary to impose the rigorous definition using two standard deviations in order to avoid any confusion of feedback effects with episodic LH secretion.

6.2 The effects of a single injection of oestradiol benzoate on LH secretion in male and female gonadectomised marmosets

6.2.a Procedure

Four male and four female gonadectomised marmosets each received a single subcutaneous injection of 35 ug ODB in 0.1ml arachis oil. Blood samples were taken immediately before the injection and at 12h intervals for 84h.

In addition, four male and four female gonadectomised animals each received a similar injection and were bled at 4h intervals for 36h. All blood samples were assayed for LH and oestradiol-17β .

Six gonadectomised animals (3 males and 3 females) received a single injection (s.c.) of 0.1ml arachis oil and were bled immediately before and 8, 20, 24, 28, 36 and 48h after the injection. Six further animals (3 males and 3 females) received no injection and were bled at the same times. These twelve animals served as controls for gonadectomised animals throughout the study.

6.2.b Results

LH levels in controls are shown in Tables 6.1 and 6.2. There was no significant difference in mean LH levels (at individual times) between males (or females) which received oil and those which did not. LH levels in oil-treated and non-treated animals have therefore been combined (Tables 6.1 and 6.2). The limits formed by two standard deviations above and below the mean control values are shown in the figures as broken lines.

The change in LH concentrations in response to a single injection of ODB in gonadectomised marmosets is shown in Figure 6.1.

ANIMAL	TIME (HOURS)						
	0	8	20	24	28	36	48
1*	48	74	57	51	69	52	63
2*	71	55	48	64	64	73	73
3*	68	60	71	68	62	49	50
Mean (n=3)	62.3	63.0	58.7	61.0	65.0	58.0	62.0
S.D.	12.5	9.8	11.6	8.9	3.6	13.0	11.5
S.E.	7.2	5.7	6.7	5.1	2.1	7.5	6.7
4	83	72	75	70	49	64	68
5	71	66	57	67	50	69	72
6	61	68	60	46	70	50	51
Mean (n=3)	71.7	68.7	64.0	61.0	56.3	61.0	63.7
S.D.	11.0	3.0	9.6	13.1	11.8	9.8	11.1
S.E.	6.4	1.7	5.6	7.5	6.8	5.7	6.4
Mean (n=6)	67.0	65.8	61.3	61.0	60.7	59.5	62.8
S.D.	11.7	7.2	9.9	10.0	9.2	10.5	10.2
S.E.	4.7	2.9	4.0	4.0	3.7	4.3	4.1
-2S.D. - +2S.D.	43.6-90.4	51.4-80.2	41.5-81.1	41.0-81.0	42.3-79.1	38.5-80.5	42.4-83.2

Table 6.1

Individual LH concentrations in control gonadectomised male marmosets, receiving arachis oil at time 0 (*), or no treatment. Means, S.E.'s, S.D.'s and the limits formed by taking two S.D.'s above and below mean values are also given

ANIMAL	TIME (HOURS)						
	0	8	20	24	28	36	48
1*	53	69	52	51	55	61	68
2*	76	62	60	48	78	59	56
3*	56	48	51	73	49	72	81
Mean (n=3)	61.7	59.7	54.3	57.3	60.7	64.0	68.3
S.D.	12.5	10.7	4.9	13.7	15.3	7.0	12.5
S.E.	7.2	6.2	2.8	7.9	8.8	4.0	7.2
4	62	46	72	56	58	80	69
5	55	59	49	67	52	62	70
6	81	66	59	64	68	59	51
Mean (n=3)	66.0	57.0	60.0	62.3	59.3	67.0	63.3
S.D.	13.5	10.1	15.5	5.6	8.1	11.4	10.7
S.E.	7.7	5.9	6.7	3.3	4.7	6.6	6.2
Mean (n=6)	63.8	58.3	57.2	59.8	60.0	65.5	65.8
S.D.	11.8	9.4	8.6	9.7	10.9	8.6	10.7
S.E.	4.8	3.8	3.5	3.9	4.5	3.5	4.3
-2S.D.-	40.2-	39.5-	40.0-	40.4-	38.2-	48.3-	44.4-
+2S.D.	87.4	77.1	74.4	79.2	81.8	82.7	87.2

Table 6.2

Individual LH concentrations in control gonadectomised female marmosets, receiving arachis oil at time 0 (*), or no treatment. Means, S.D.'s, S.E.'s and the limits formed by taking two S.D.'s above and below mean values are also given

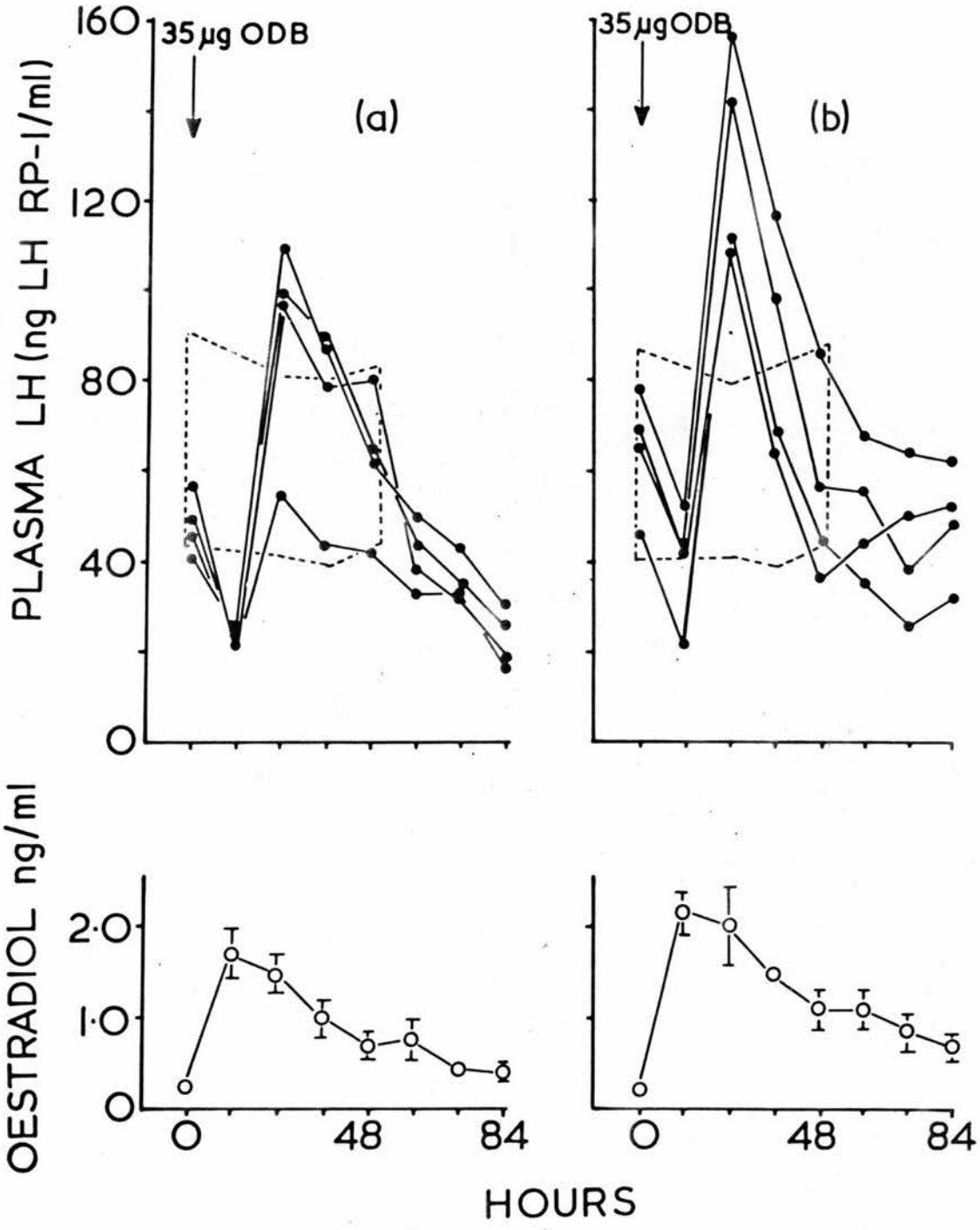


Figure 6.1

LH and oestradiol concentrations in gonadectomised males (a) and females (b) after a single s.c. injection of 35ug ODB in oil

Individual results for LH; oestradiol values are Mean ±S.E.M.(n=4). Throughout this chapter the dotted areas represent the limits formed by 2 S.D.'s above and below the mean control values.

A biphasic response with an initial suppression of LH secretion followed by an abrupt increase in LH secretion can be clearly seen. In all females LH levels were reduced 12h after the oestrogen injection, although in only one case did levels fall below the defined control limits. Following the suppression of LH secretion there was a marked increase of LH between 12 and 24h after the oestrogen injection, and a positive feedback response was observed in all females. Maximum LH levels occurred after 24h, after which LH secretion declined rapidly. A similar pattern of LH release was seen in gonadectomised male marmosets. An initial suppression of LH occurred after 12h (all males showed negative feedback), after which LH secretion increased to reach a peak after 24h. Only three males, however, showed positive feedback. Circulating oestradiol levels achieved by the injection were approximately 2.0 ng/ml after 12h and declined progressively thereafter. Oestradiol concentrations were similar in males and females. Figure 6.2(a) compares the mean LH levels in males and females with their respective controls. In both males and females mean LH concentrations 24h after the injection of oestrogen were significantly higher than control values. With the exception of levels after 12h there was no significant difference in mean LH concentrations between males and females throughout the 84h period (Fig. 6.2(b)).

The effect of a single injection of ODB on LH concentrations over a 36h period is shown in Figure 6.3. The patterns of LH secretion are similar to those shown previously (Fig. 6.1). As plasma oestradiol concentrations rose LH levels initially fell and remained suppressed between 4 and 12h after the injection. LH levels fell below two standard deviations below mean control values (i.e. negative feedback)

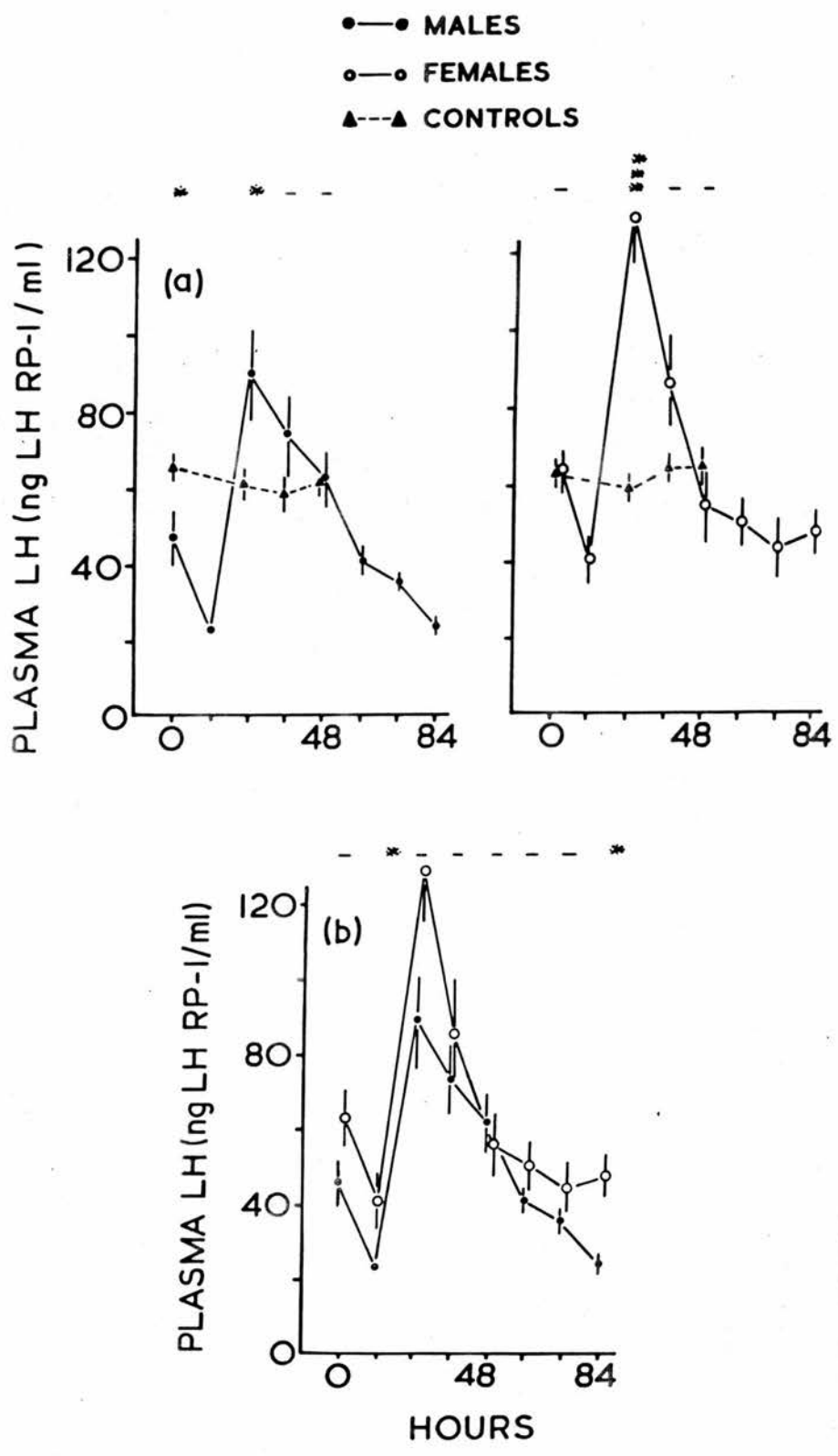


Figure 6.2

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6).

The following notation will apply throughout this chapter:-

-, N.S.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with controls. (Student's t-test).

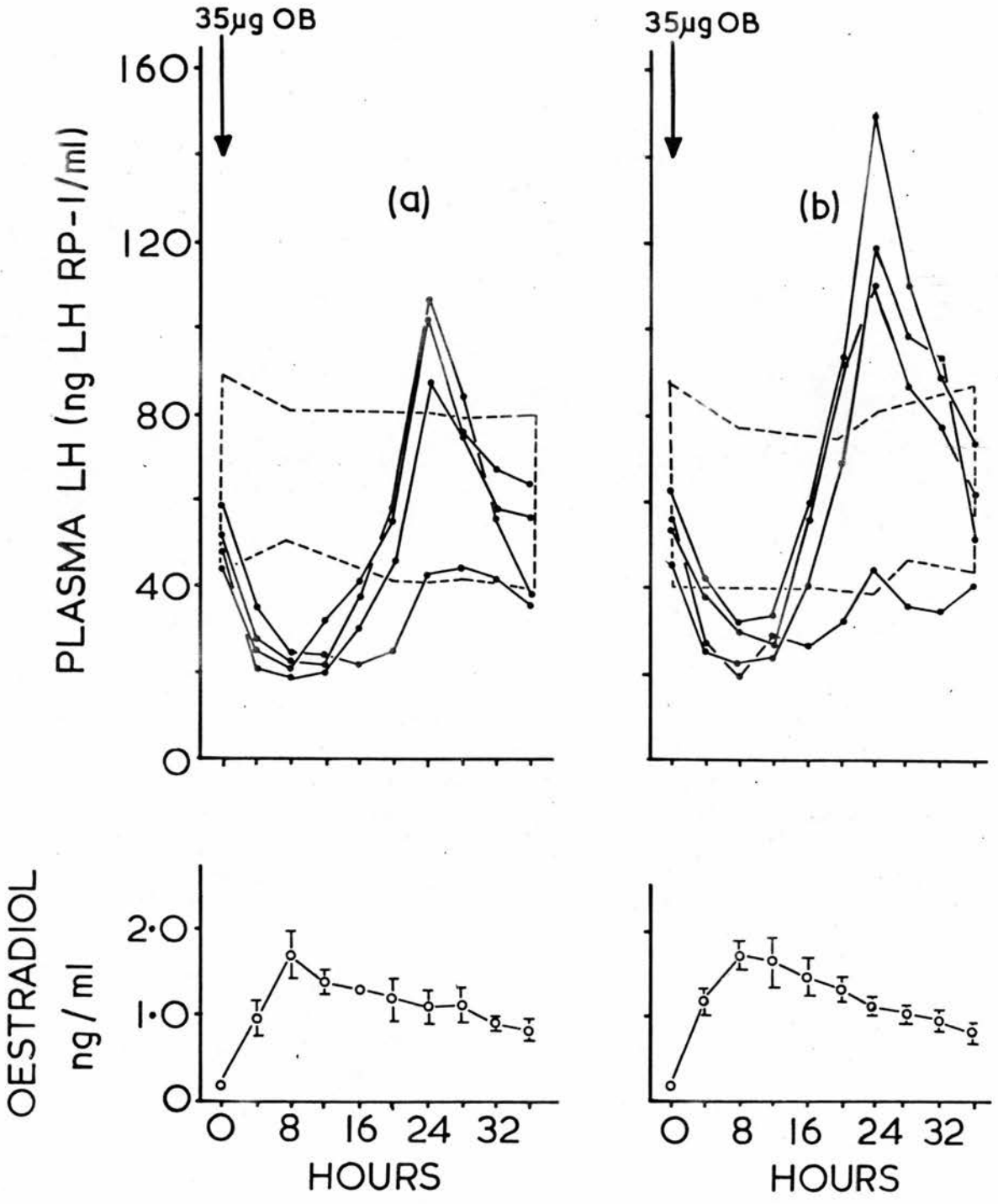


Figure 6.3

LH and oestradiol concentrations in gonadectomised males (a) and females (b) after a single injection of 35ug ODB in oil

Individual results for LH; oestradiol values are Mean ±S.E.M. (n=4).

in all animals after 8h, and three males and three females showed positive feedback,with maximum LH levels occurring after 24h. Although there was an increase in LH secretion following the initial negative response in the remaining two animals, this is not interpreted as clear positive feedback. Maximum observed oestradiol-17β concentrations (approximately 1.75 ng/ml) occurred 8h after the injection. In all animals mean LH levels 8h after the ODB injection were significantly lower than mean control levels, whereas after 24h mean LH concentrations were significantly higher than mean control values only in females (Fig. 6.4(a)). There was, however, no significant difference in the LH response between males and females (Fig. 6.4(b)).

6.3 The effects of a single injection of oestradiol benzoate on LH secretion in intact male marmosets

6.3.a Procedure

Sixteen intact males received single injections (s.c.) of 35ug ODB in 0.1ml arachis oil and were bled immediately before and 8, 20, 24, 28, 36 and 48h after the injection. Blood samples from ten animals were assayed for LH and oestradiol-17β (Group 1) and samples from the remaining six animals were assayed for LH, oestradiol-17β and testosterone (Group 2).

Six intact males received a single injection of 0.1ml arachis oil and six animals received no injection. Blood samples, collected as above, were assayed for LH, and served as controls.

6.3.b Results

LH concentrations in controls are shown in Table 6.3. There was no significant difference in mean LH levels (at individual times)

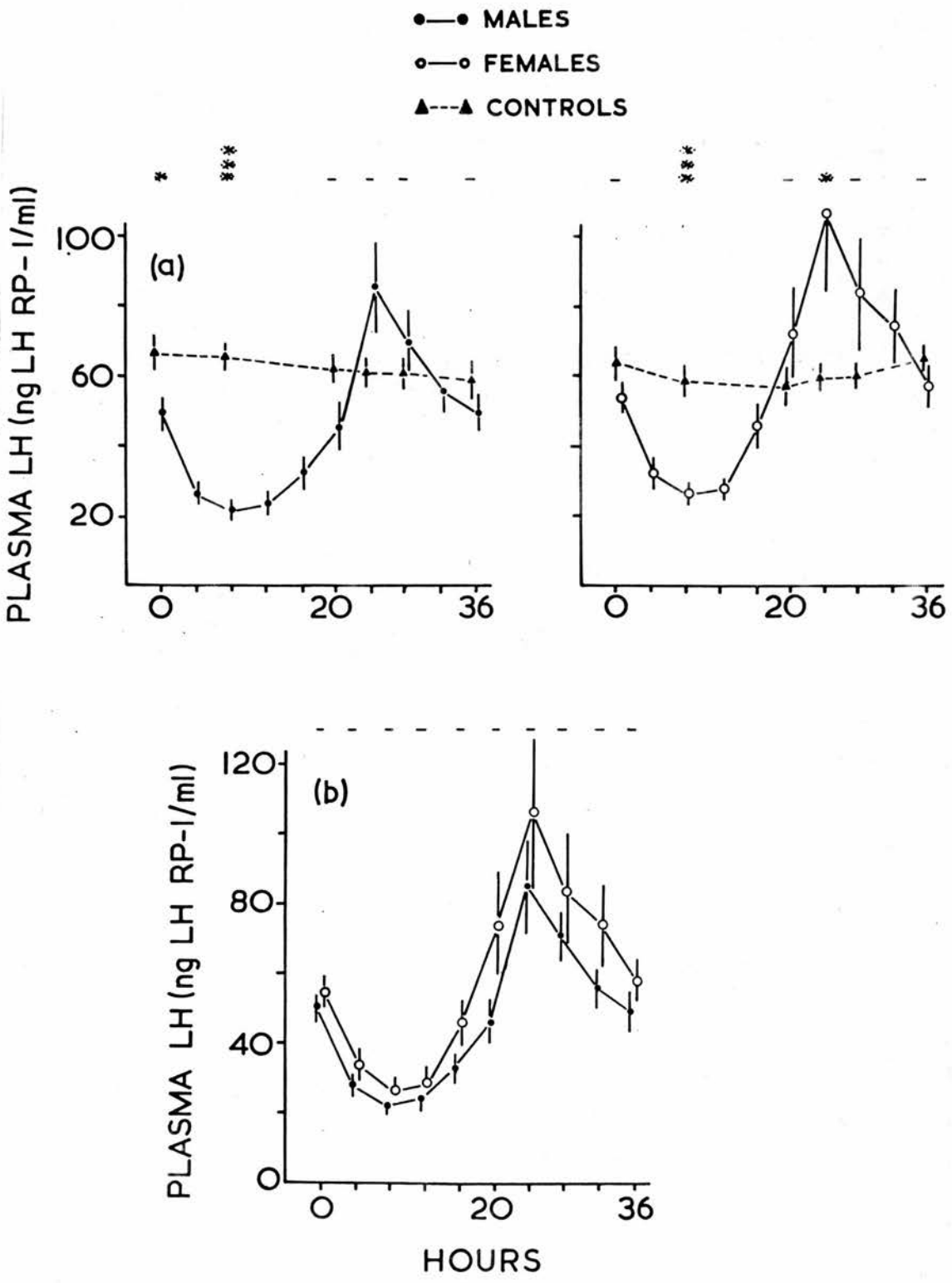


Figure 6.4

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6)

ANIMAL	TIME(HOURS)						
	0	8	20	24	28	36	48
1*	29	46	47	47	27	44	35
2*	28	34	31	20	27	52	41
3*	36	38	41	55	60	46	56
4*	43	36	42	27	20	31	48
5*	69	62	30	34	42	61	57
6*	36	54	52	43	31	27	31
Mean (n=6)	40.2	45.0	40.5	37.7	37.3	43.5	44.7
SD	15.1	11.1	8.7	13.1	13.6	12.8	10.8
SE	6.2	4.6	3.5	5.3	5.6	5.2	4.4
1	46	31	54	61	59	49	37
2	63	59	37	41	35	66	49
3	38	43	41	37	29	39	36
4	21	41	34	43	31	42	41
5	72	61	42	47	57	54	68
6	49	46	32	29	49	32	38
Mean (n=6)	48.2	46.8	40.0	43.0	43.3	47.0	44.8
SD	18.1	11.4	7.9	10.7	13.3	12.0	12.3
SE	7.3	4.7	3.2	4.4	5.5	4.9	5.0
Mean (n=12)	44.2	45.9	40.3	40.3	40.3	45.2	44.7
SD	16.4	10.8	7.9	11.7	13.2	12.0	11.0
SE	4.7	3.1	2.3	3.4	3.8	3.5	3.2
-2 SD -	11.4-	24.3-	24.5-	16.9-	13.9-	21 -	22.7-
+2 SD	77	67.5	56.1	63.7	66.7	69.2	66.7

Table 6.3

Individual LH concentrations in control intact male marmosets, receiving arachis oil at time 0 (*), or no treatment. Means, S.E.'s, S.D.'s and the limits formed by taking 2 S.D.'s above and below mean values are also given

between males which received oil and those which did not. LH levels in oil-treated and non-treated animals have therefore been combined.

The change in LH secretion in group 1 animals in response to oestrogen is shown in Figure 6.5. A decrease in LH secretion was observed 8h after oestrogen administration in eight animals, although this decrease could be classified as negative feedback in only four animals. In two animals LH levels were undetectable before and 8h after the oestrogen injection and therefore no change in LH secretion could be observed. Following the initial suppression of LH, five animals responded with a clear positive discharge of LH, maximum LH levels occurring after 28-36h. In the other five animals the maximum LH levels attained remained within the defined control limits. Maximum observed oestradiol levels, with a mean value of approximately 1.8ng/ml, were attained 8h after the injection. Compared with mean control values, mean LH levels in animals receiving ODB were significantly lower after 8h and significantly higher after 28h (Fig. 6.6).

LH levels in animals in group 2 are shown in Figure 6.7. LH levels were suppressed after 8h in all animals although this suppression could be classified as negative feedback only in one animal. In the four animals which showed positive feedback maximum LH levels were attained after 28h. The elevated levels of oestradiol caused a suppression of testosterone secretion and circulating levels were markedly reduced 8h after the injection. The length of time for which testosterone levels were reduced cannot be determined from the bleeding schedule used, although levels had returned to pre-injection values 24h after the injection. Compared with mean control values, mean LH

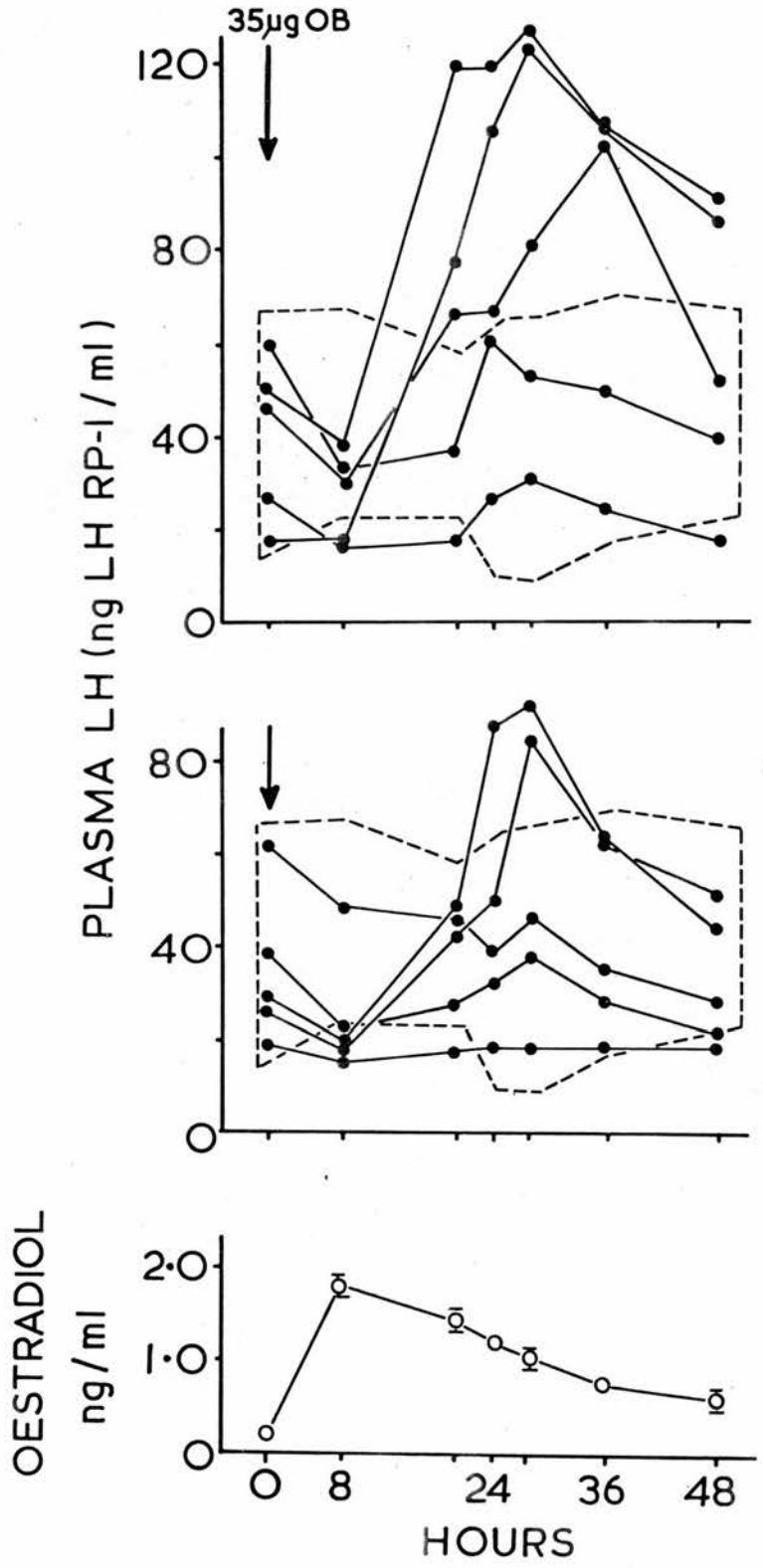


Figure 6.5

LH and oestradiol concentrations in intact males after a single s.c. injection of 35 ug ODB in oil

Individual results for LH are shown in 2 graphs; oestradiol values are Mean ± S.E.M. (n=10).

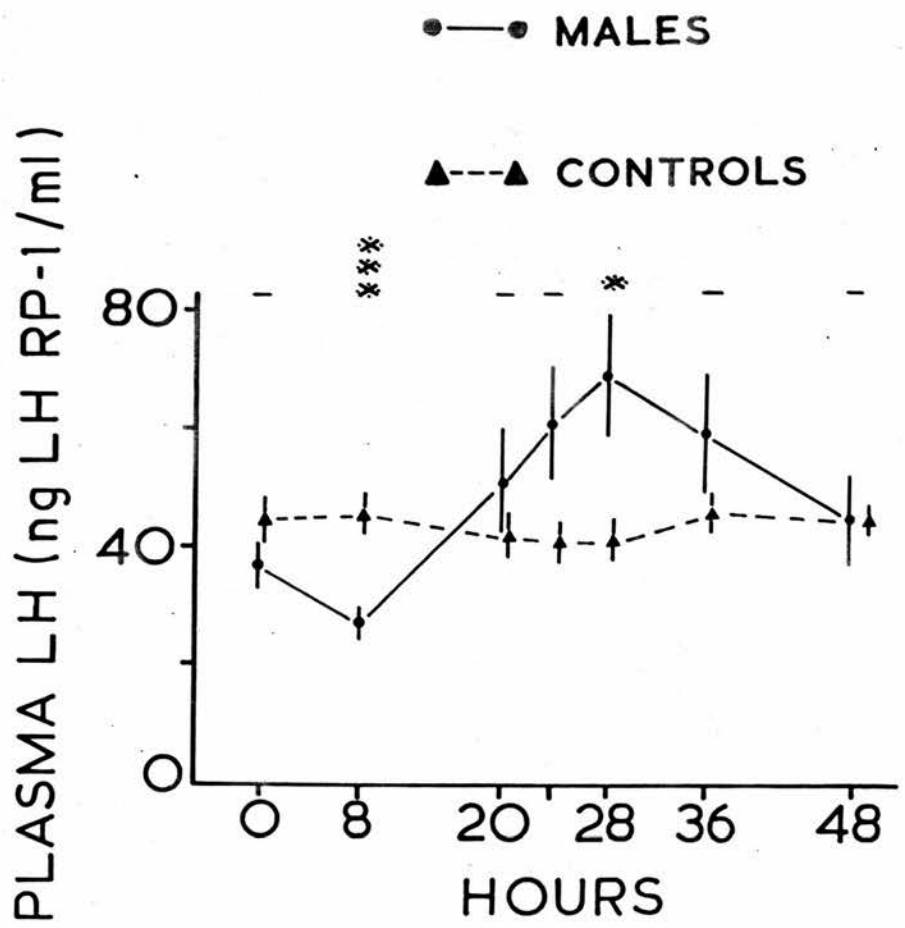


Figure 6.6

Mean (± S.E.M.) LH concentrations in intact males (n=10) and controls (n=12)

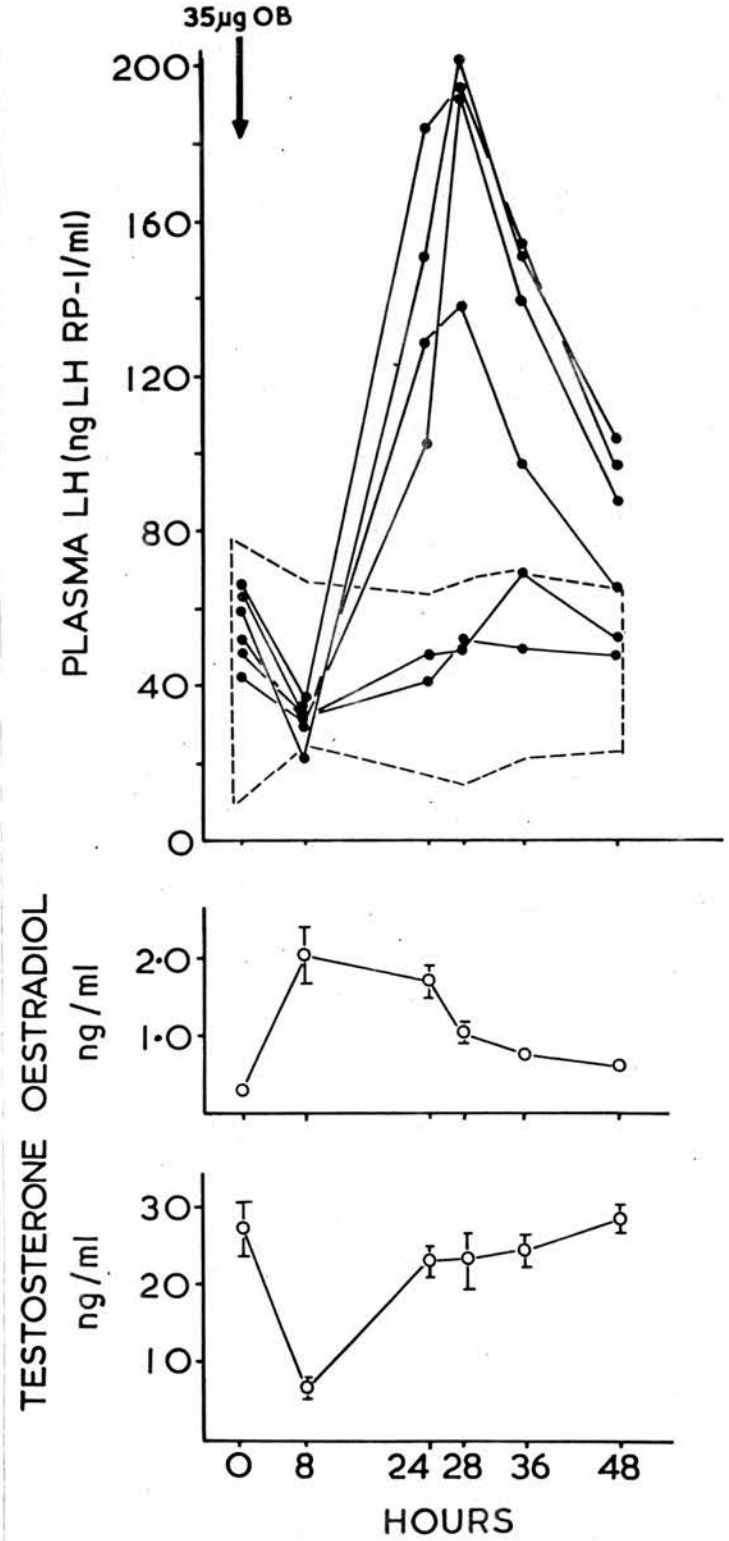


Figure 6.7

LH, oestradiol and testosterone concentrations in intact males after a single s.c. injection of 35ug ODB in oil

Individual results for LH; oestradiol and testosterone values are Mean $\pm$ S.E.M. (n=6).

levels in animals receiving ODB were significantly lower after 8h and significantly higher after 24, 28, 36 and 48h (Fig. 6.8). Mean LH concentrations in all sixteen intact males were not significantly different from those in gonadectomised females (Fig. 6.4) at any time after the injection of oestrogen (Fig. 6.9)

6.4 The effects of testosterone, dihydrotestosterone and progesterone on oestradiol induced LH release in gonadectomised marmosets

6.4.a Procedure

Three groups of gonadectomised marmosets, each comprising four males and four females were given a single injection (s.c.) of 35ug ODB in 0.1ml arachis oil, followed immediately and after 8h by a single injection (s.c.) of 0.5mg T/injection (group 1), 0.5mg DHT/injection (group 2) or 1.0mg P/injection (group 3) in 0.1ml arachis oil. A fourth group (four males and four females) received progesterone implants (1 x 50mg plus 1 x 25mg) 8 days before the oestradiol injections. Blood samples were taken immediately before and 8, 20, 24, 28, 36 and 48h after the oestrogen injections, except that no 48h samples were taken in groups 3 and 4. The doses of steroids used were chosen to give circulating concentrations within the physiological range.

6.4.b Results

Group 1

LH concentrations after oestradiol and testosterone injections are shown in Figures 6.10 and 6.11. There was an initial fall (after 8h) in LH levels in all animals although only three males and three females showed clear negative feedback. All females showed positive

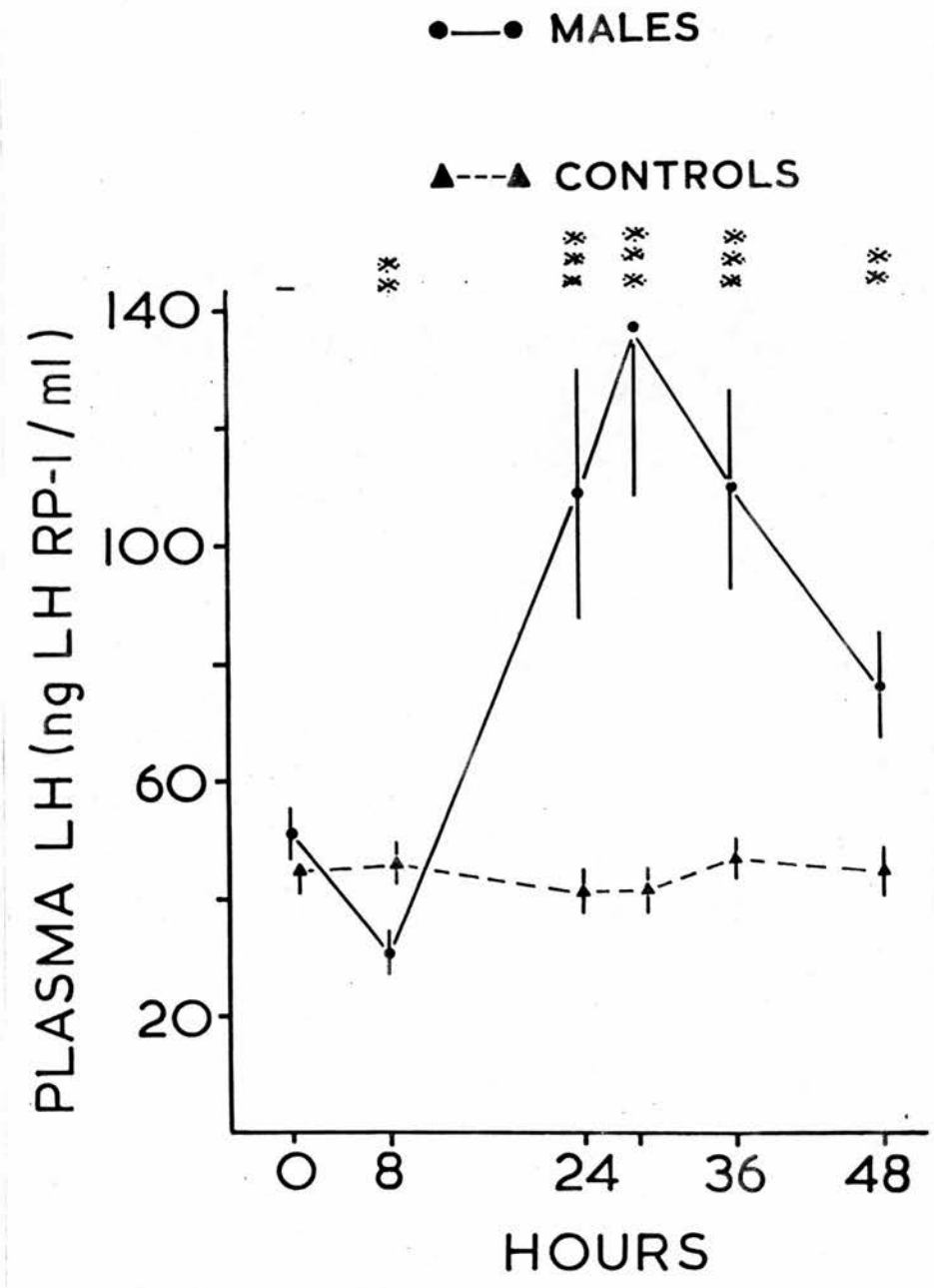


Figure 6.8

Mean (± S.E.M.) LH concentrations in intact males (n=6) and controls (n=12)

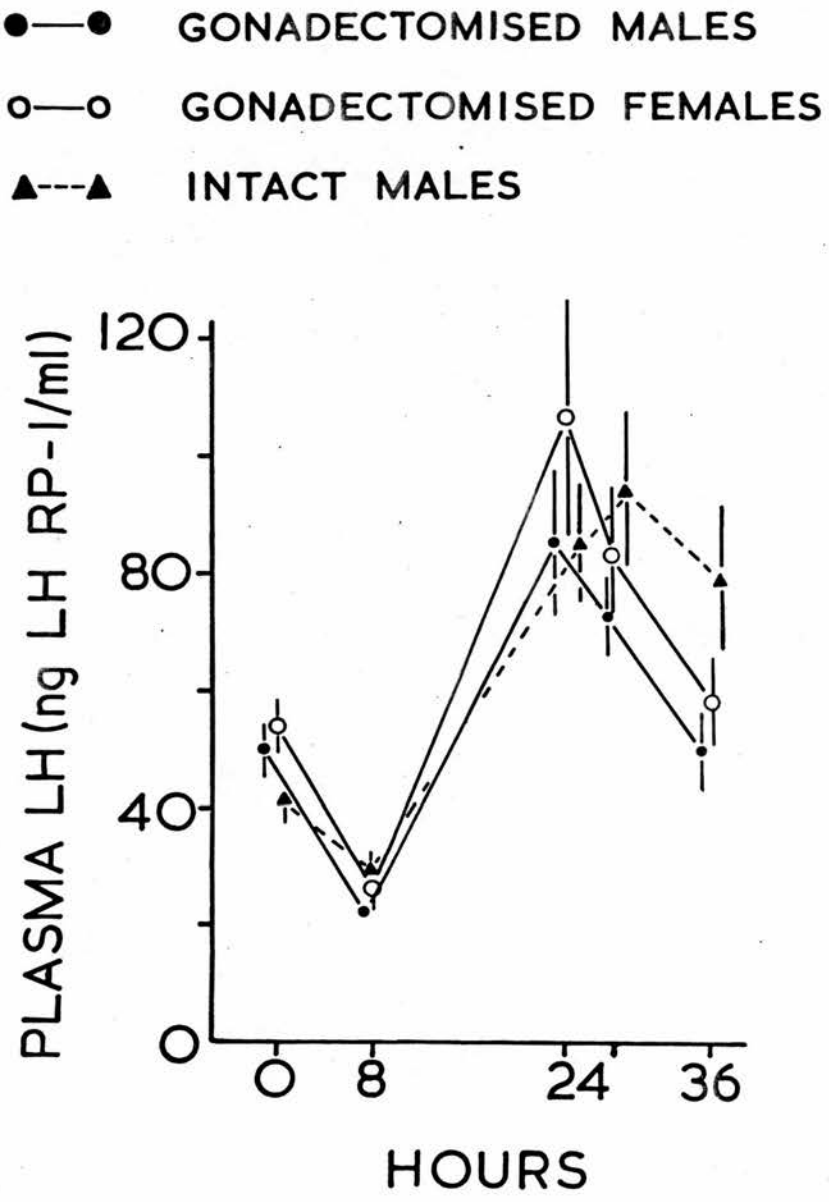


Figure 6.9

Mean (± S.E.M.) LH concentrations in intact males (n=16), and gonadectomised males (n=4) and females (n=4)

There were no significant differences between the groups at any time after the injection of ODB.

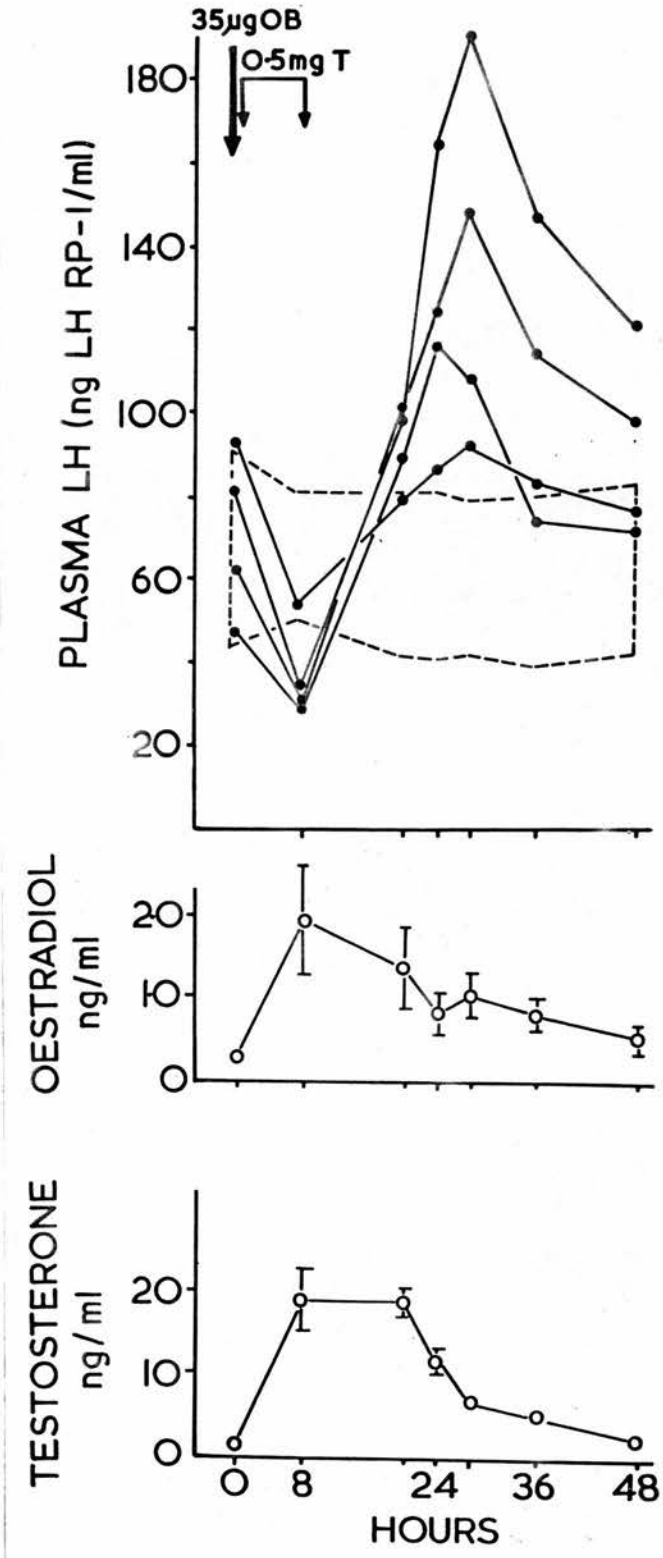


Figure 6.10

LH, oestradiol and testosterone concentrations in gonadectomised males after a single s.c. injection of 35ug ODB in oil and s.c. injections of 0.5mg T in oil after 0 and 8 h.

Individual results for LH; oestradiol and testosterone values are Mean $\pm$ S.E.M. (n=4).

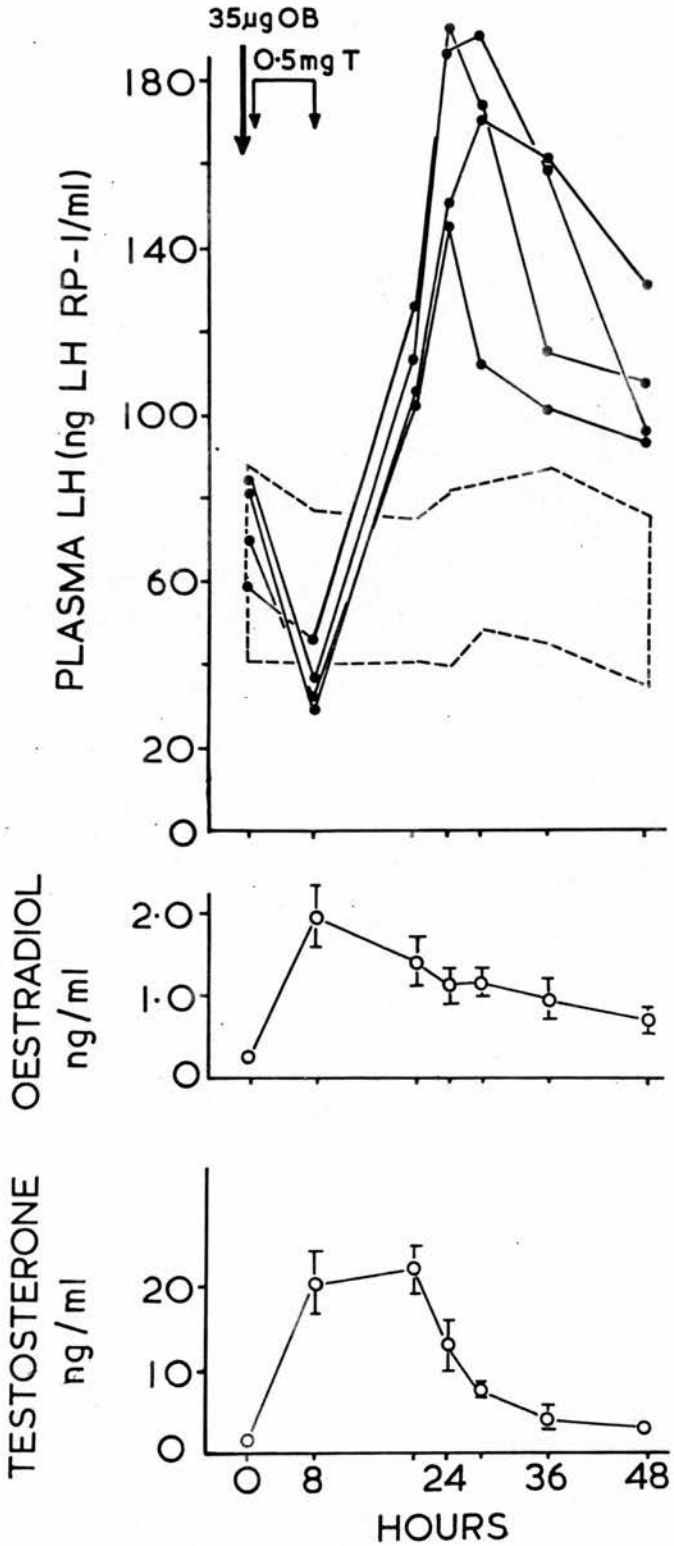


Figure 6.11

LH, oestradiol and testosterone concentrations in gonadectomised females after a single s.c. injection of 35ug ODB in oil and s.c. injections of 0.5mg T in oil after 0 and 8h.

Individual results for LH; oestradiol and testosterone values are Mean $\pm$ S.E.M. (n=4).

feedback after 24h although in one animal maximum LH levels occurred after 28h. Maximum LH levels exceeded two standard deviations above mean control values in all males but in one case the pre-injection level was also above the control limits. In three males maximum LH levels were attained after 28h whereas in one animal LH levels were highest after 24h. Mean oestradiol levels of approximately 2.0ng/ml were reached after 8h and were similar to the levels obtained in the absence of testosterone (c.f. Figs. 6.1 and 6.3). Mean testosterone levels were similar in males and females, with maximum concentrations (approximately 20ng/ml) occurring between 8 and 20h after the first injection. Figure 6.12(a) compares mean LH concentrations in experimental animals with those of the controls. LH levels were significantly lower than control values after 8h and significantly higher than control values after 20, 24, 28, 36 and 48h in both males and females. The response in females did not differ significantly from the response in males, except at 20h after the oestrogen injection (Fig. 6.12(b)). Thus, under the experimental conditions described testosterone does not appear to inhibit oestrogen induced positive feedback.

Group 2

Negative feedback occurred after 8h in all males and in three females (Fig. 6.13). The level of LH was considerably reduced in the remaining female although it did not fall outside the defined control limits. All males showed positive feedback and maximum LH levels were attained after 24h. Three females showed a positive discharge of LH and maximum LH levels occurred after 24h in two animals and after 28h in one animal. Oestradiol-17β levels were similar to those in group 1. Mean LH concentrations in animals in group 2

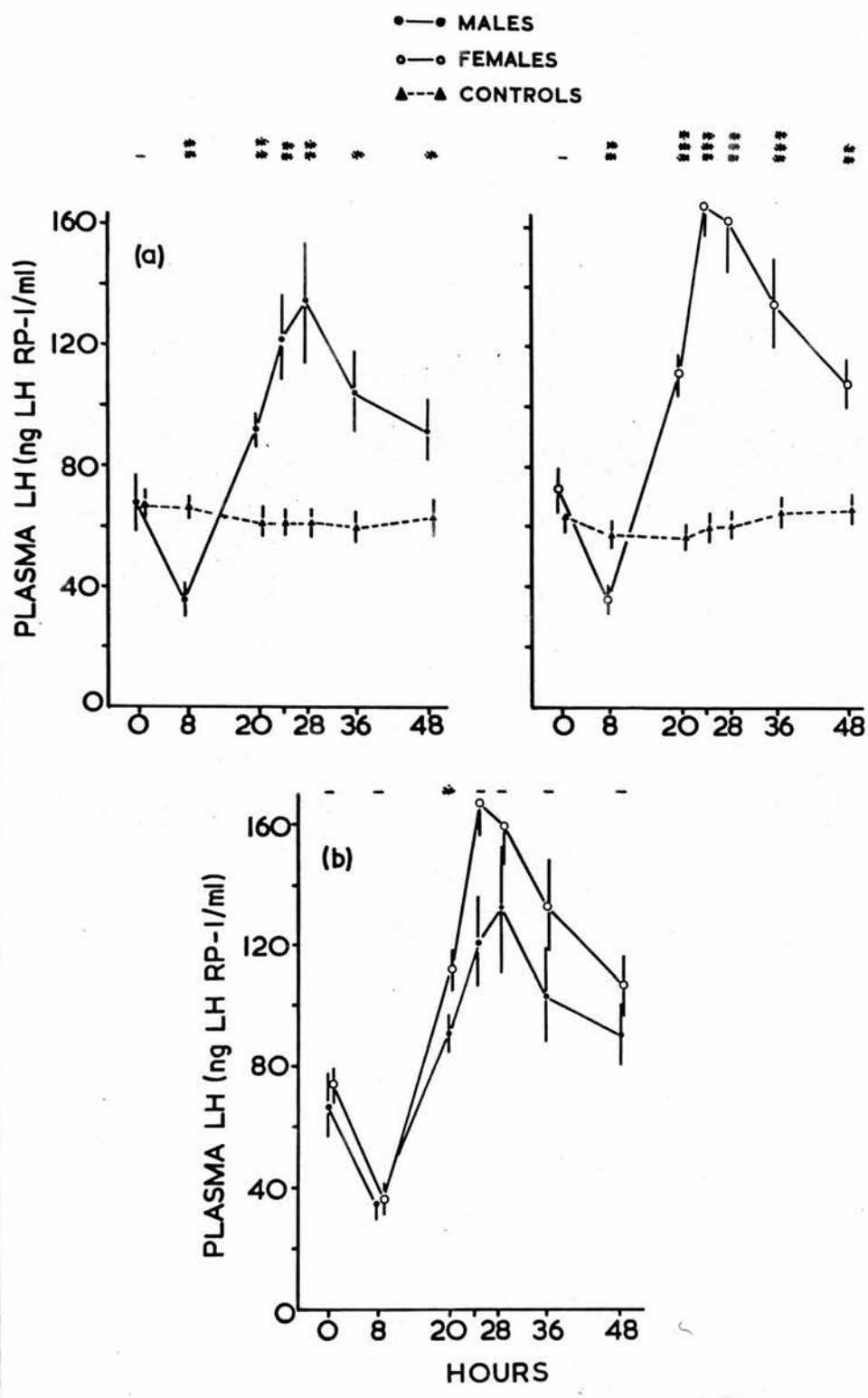


Figure 6.12

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6)

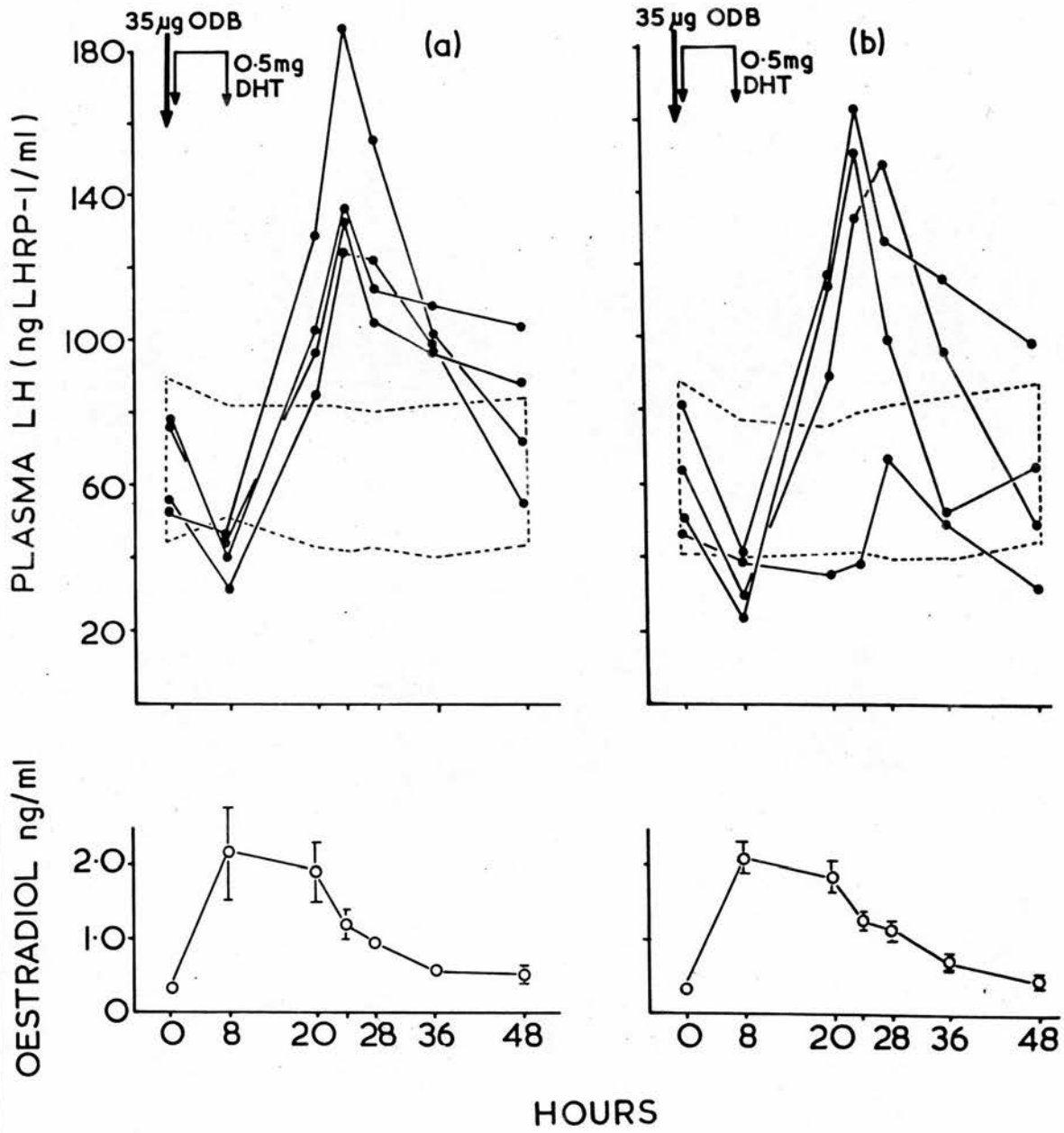


Figure 6.13

LH and oestradiol concentrations in gonadectomised males (a) and females (b) after a single s.c. injection of 35ug ODB in oil and s.c. injections of 0.5mg DHT in oil after 0 and 8h

Individual results for LH; oestradiol values are Mean ± S.E.M. (n=4).

are compared with mean control values in Figure 6.14(a). LH levels in males and females were significantly lower than control values after 8h. LH levels were significantly higher than control values after 20, 24, 28 and 36h in males and after 24 and 28h in females. There were no significant differences in mean LH levels between males and females (Fig. 6.14(b).

Group 3

All animals showed negative followed by positive feedback (Figure 6.15). Apart from two males and one female in which LH levels were highest after 28h, maximum LH concentrations occurred after 24h. Oestradiol levels were similar to those obtained in all previous experiments. The injections of progesterone achieved circulating levels of approximately 35ng/ml between 8 and 20h after the first injection, after which levels declined fairly rapidly. Mean LH concentrations in males and females were significantly lower after 8h and significantly higher thereafter compared with mean control values (Fig. 6.16(a)). There was no significant difference in mean LH concentrations between males and females, after the oestrogen injection (Fig. 6.16(b).

Group 4

All animals showed negative feedback 8h after the injection of oestrogen (Figure 6.17). However, maximum LH levels exceeded two standard deviations above mean control values only in one male (after 28h) and one female (after 20h). Oestradiol concentrations were similar to those obtained in previous experiments. Although progesterone concentrations varied throughout the experiment (more so in the females) the levels achieved by the implants (approximately 35-40ng/ml) were similar to those produced by the

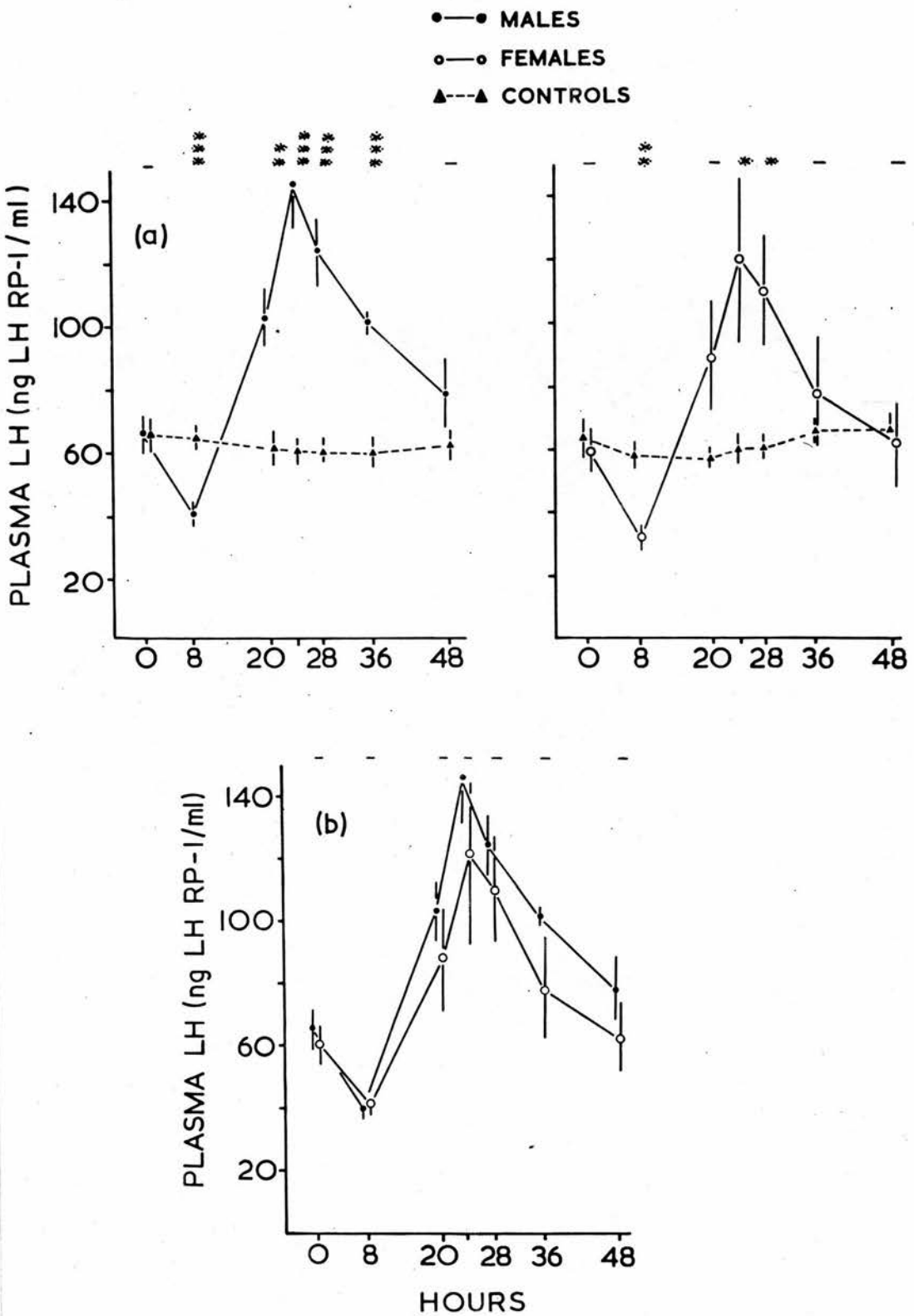


Figure 6.14

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6).

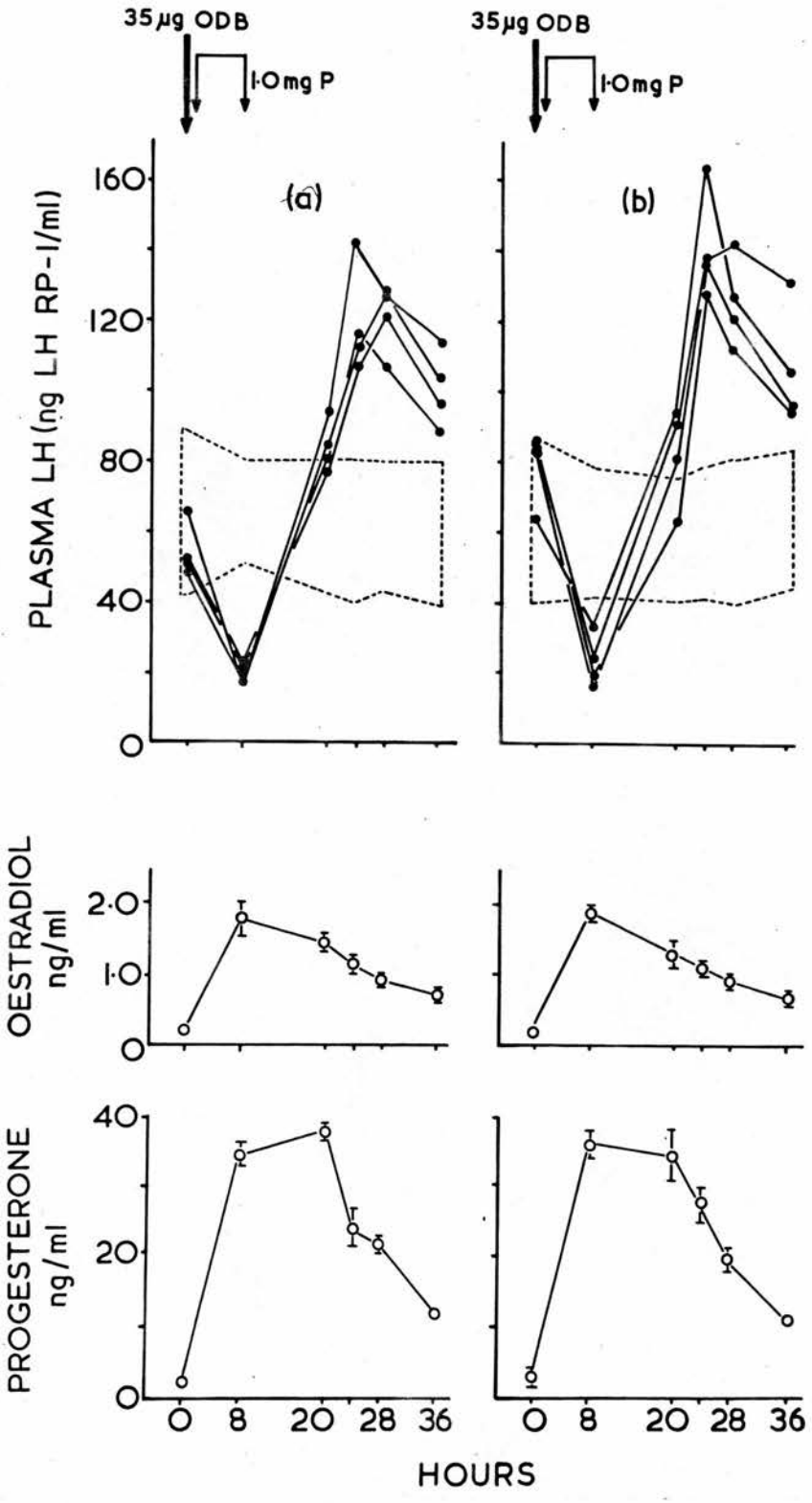


Figure 6.15

LH, oestradiol and progesterone concentrations in gonadectomised males (a) and females (b) after a single s.c. injection of 35ug ODB in oil and s.c. injections of 1.0mg P in oil after 0 and 8h.

Individual results for LH; oestradiol and progesterone values are Mean $\pm$ S.E.M. (n=4).

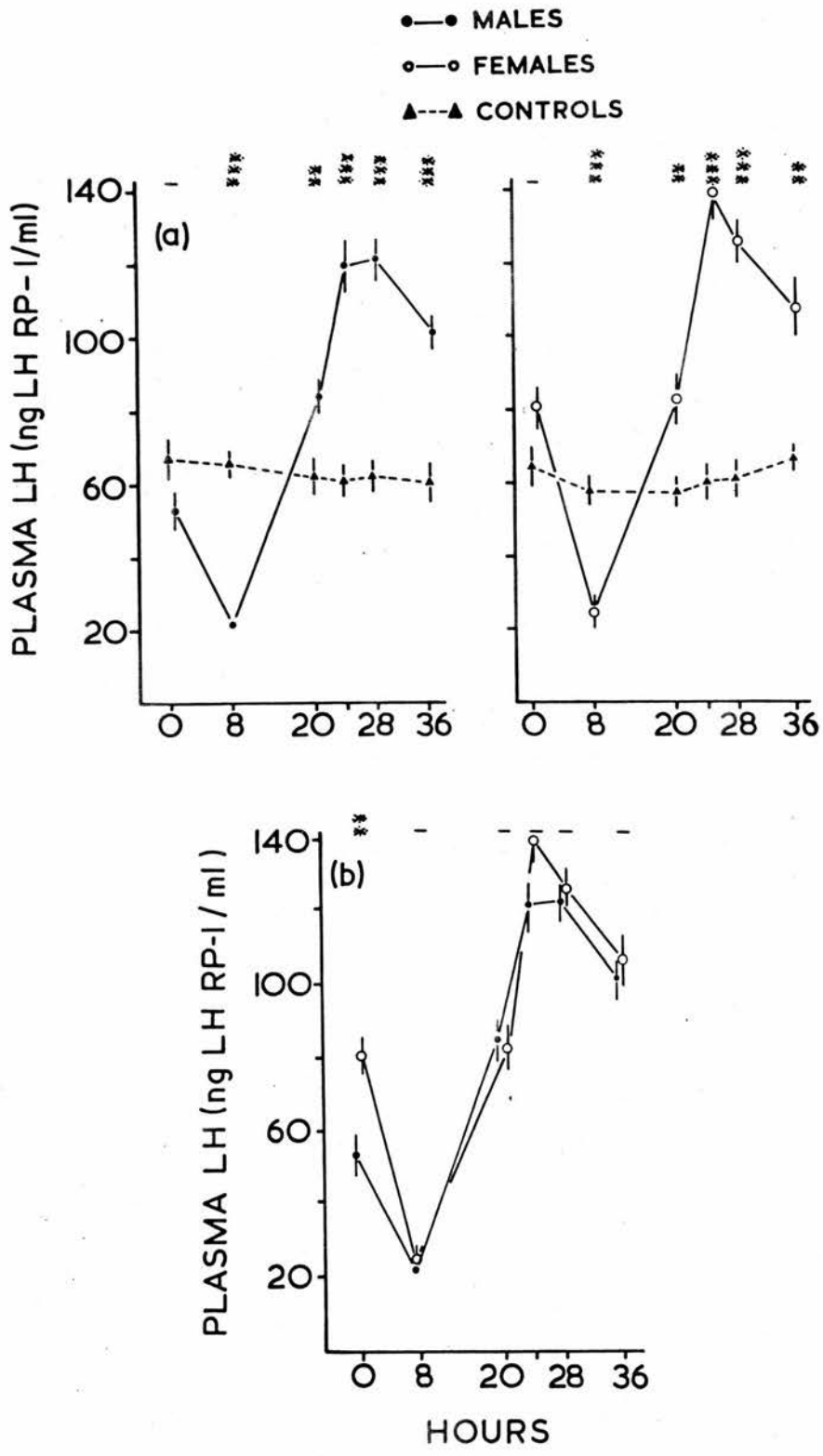


Figure 6.16

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6).

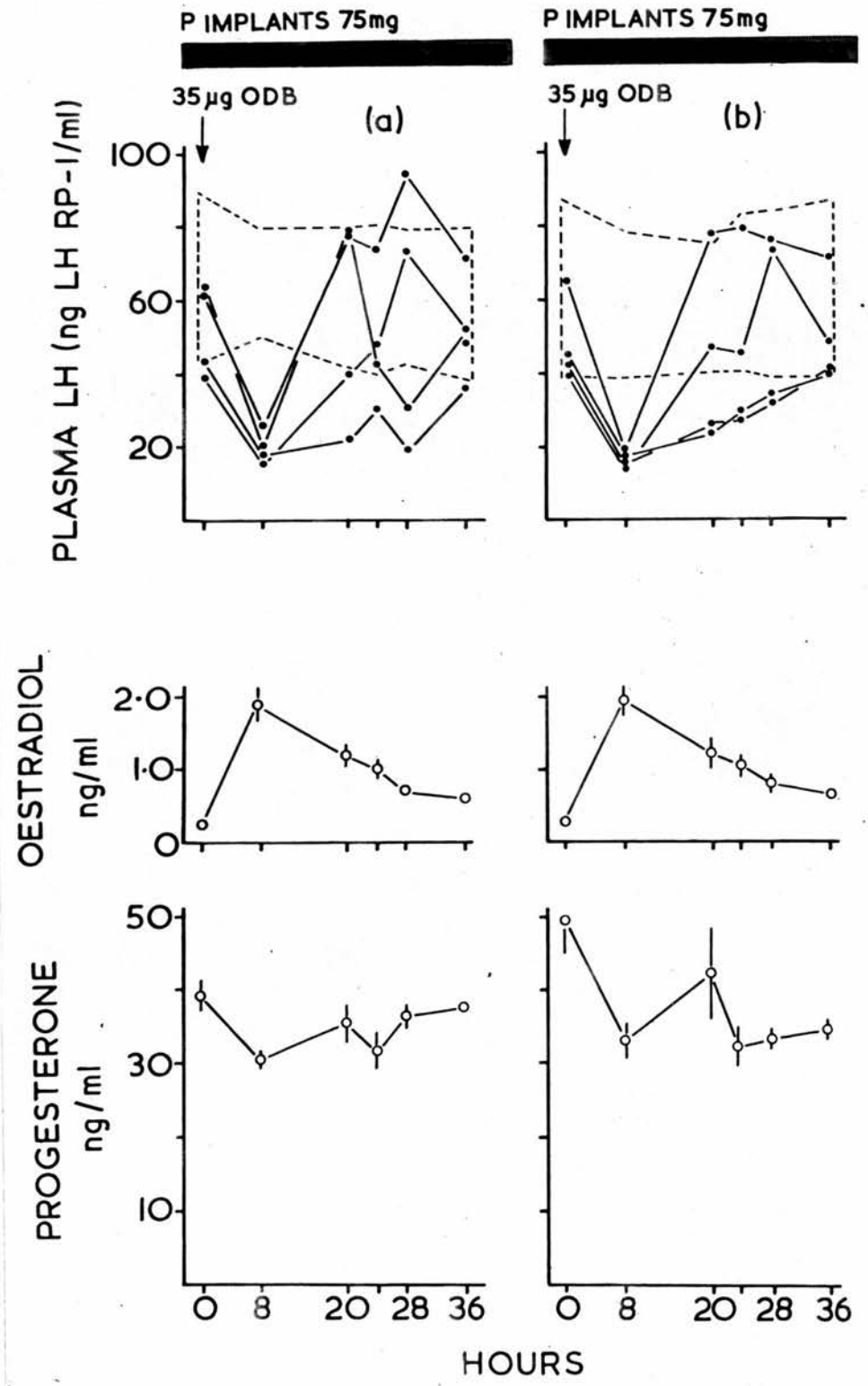


Figure 6.17

LH, oestradiol and progesterone concentrations in gonadectomised males (a) and females (b) after a single s.c. injection of 35ug ODB in oil to animals containing 75mg progesterone implants

Individual results for LH; oestradiol and progesterone values are Mean $\pm$ S.E.M. (n=4).

injections (group 3). Mean LH concentrations in males and females were significantly lower than mean control values after 8h, but thereafter there was no significant difference between control and experimental animals (Fig. 6.18(a)). There was also no significant difference in the LH response between males and females (Fig. 6.18(b).

6.5 The effects of injections of testosterone, dihydrotestosterone and progesterone on LH release in gonadectomised marmosets

6.5.a Procedure

Two groups of gonadectomised marmosets each comprising three males and three females were given two subcutaneous injections of either 2.5mg T/injection (group 1) or 2.5mg DHT/injection (group 2) in 0.1ml arachis oil at time 0 and after 8h. Each animal was bled immediately before and 8, 20, 24, 28 and 36h after the first injection. In addition two groups (groups 3 and 4) of gonadectomised marmosets (three females per group) were given two subcutaneous injections of 2.0mg P/injection in 0.1 ml arachis oil at time 0 and after 8h. Animals in group 4 were implanted with oestradiol capsules 8 days before the injections. Animals were bled as in groups 1 and 2 except that an extra blood sample was collected after 48h.

6.5.b Results

Group 1

Testosterone failed to induce negative feedback in any of the animals (Fig. 6.19). None of the females showed a positive discharge of LH although positive feedback occurred in two of the males after 24h. Circulating levels of testosterone obtained with the injections were similar in males and females and levels of approximately

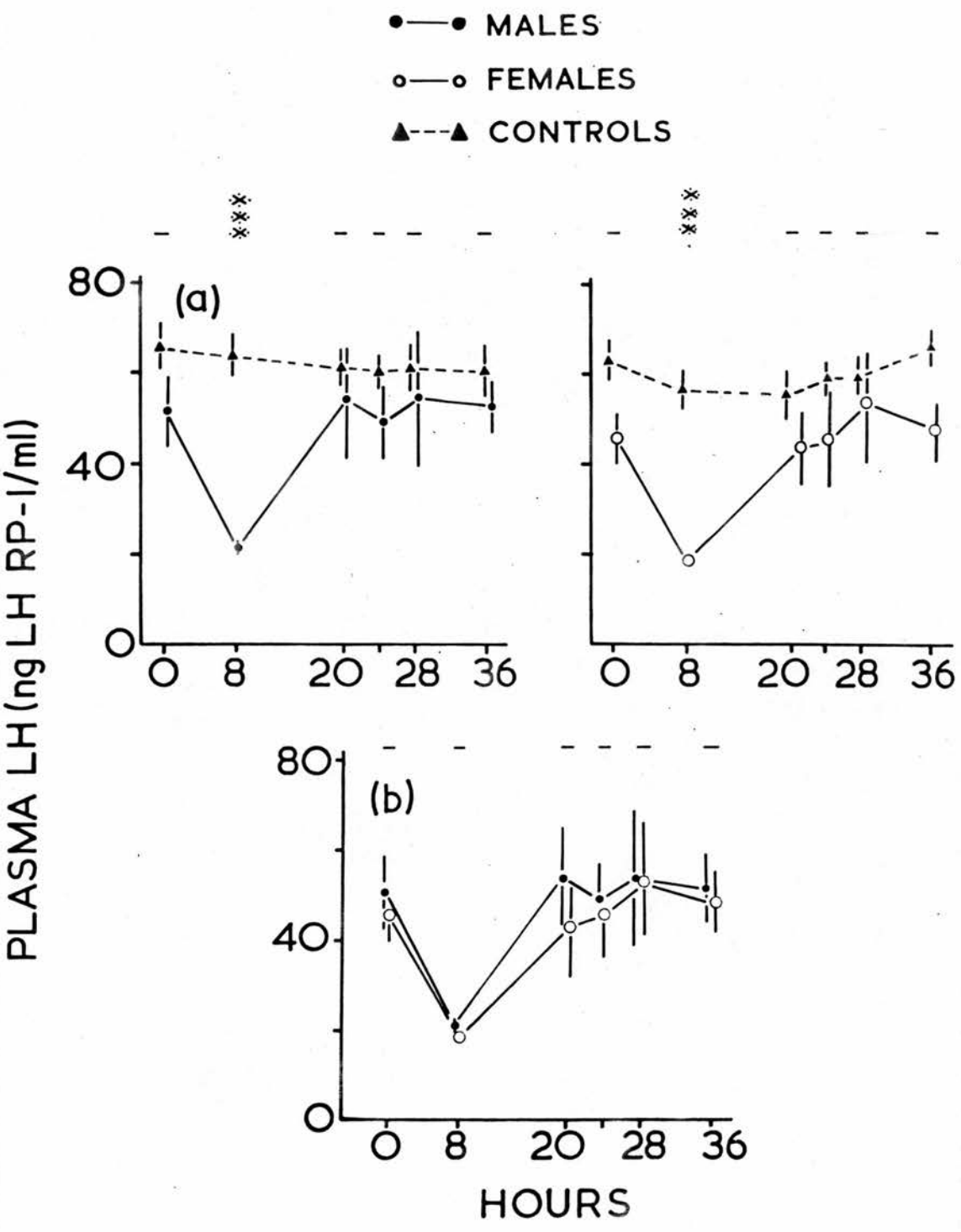


Figure 6.18

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=4) and females (n=4) and in respective controls (males and females, n=6).

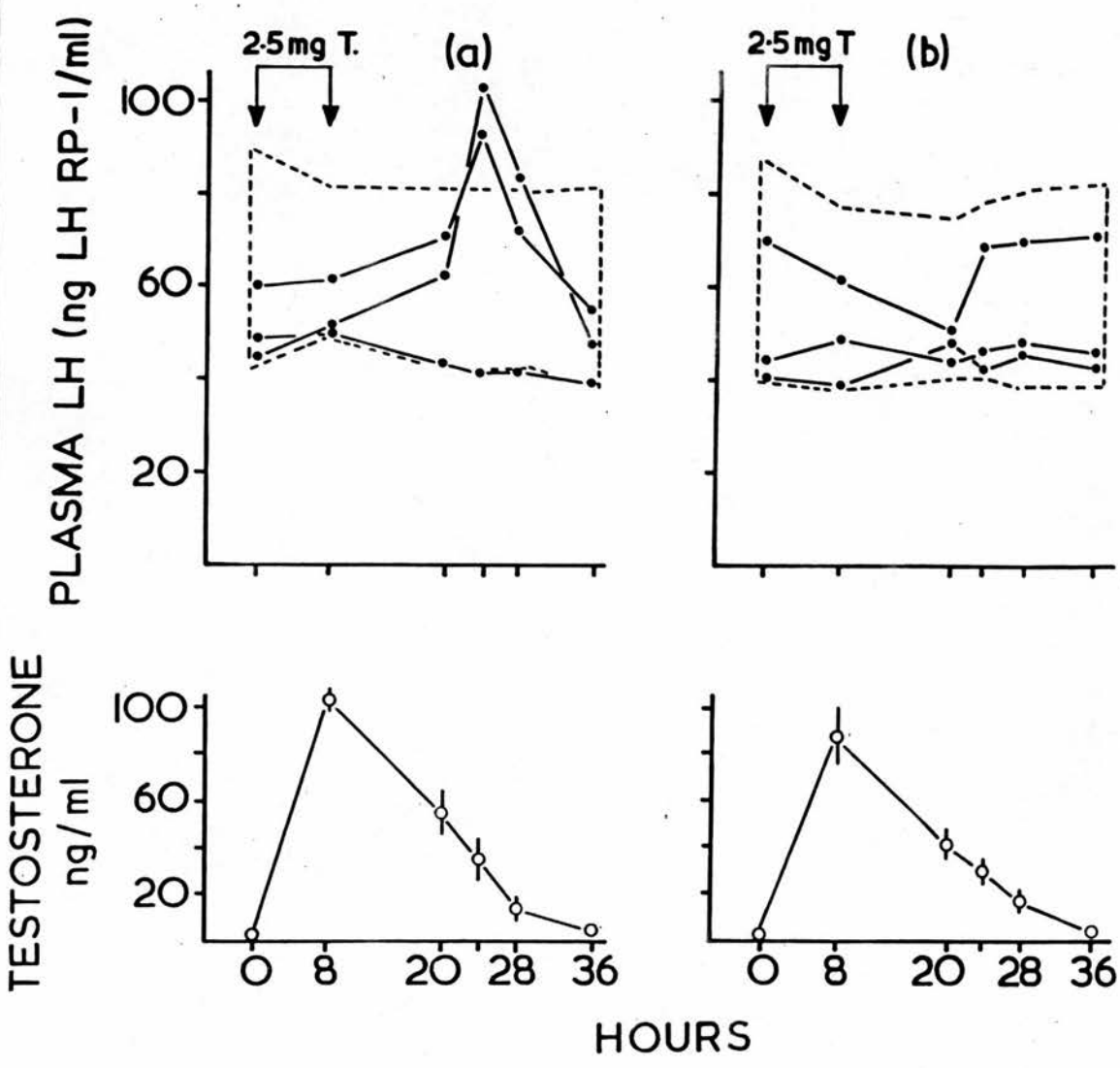


Figure 6.19

LH and testosterone concentrations in gonadectomised males (a) and females (b) after s.c. injections of 2.5mg T in oil at 0 and 8h.

Individual results for LH; testosterone values are Mean ± S.E.M. (n=3).

90-100 ng/ml were recorded after 8h. It is likely that the second injection of testosterone produced a further increase in circulating testosterone levels but there were no blood samples to show this. There were no significant differences either in mean LH levels in males and females compared with control values (Fig. 6.20(a)) or between male and female responses (Fig. 6.20(b)).

Group 2

Injections of DHT did not induce negative or positive feedback in any of the animals (Fig. 6.21). . There was no significant difference in mean LH levels between experimental and control animals (Fig. 6.22(a)), and the response in males did not differ significantly from the response in females (Fig. 6.22(b).

Groups 3 and 4

Females receiving progesterone only did not show negative or positive feedback (Fig. 6.23) . In the presence of oestrogen however, similar concentrations of progesterone (maximum observed levels were approximately 80-90 ng/ml) produced a clear increase in LH concentrations in two of the animals (Fig. 6.24). Maximum LH levels were attained 24h after the first injection. Circulating levels of oestradiol achieved by the implants were of the order of 0.5 ng/ml. As the control values are not applicable to animals in group 4, where LH levels have been chronically suppressed with oestrogen, the standard definition of positive feedback cannot be used in this case. Nevertheless a marked increase in LH secretion could clearly be seen in two animals. As LH secretion was already chronically suppressed with oestrogen implants, no further negative feedback action could be observed. Progesterone therefore was seen to induce an increase in LH secretion, but only in the presence of oestrogen.

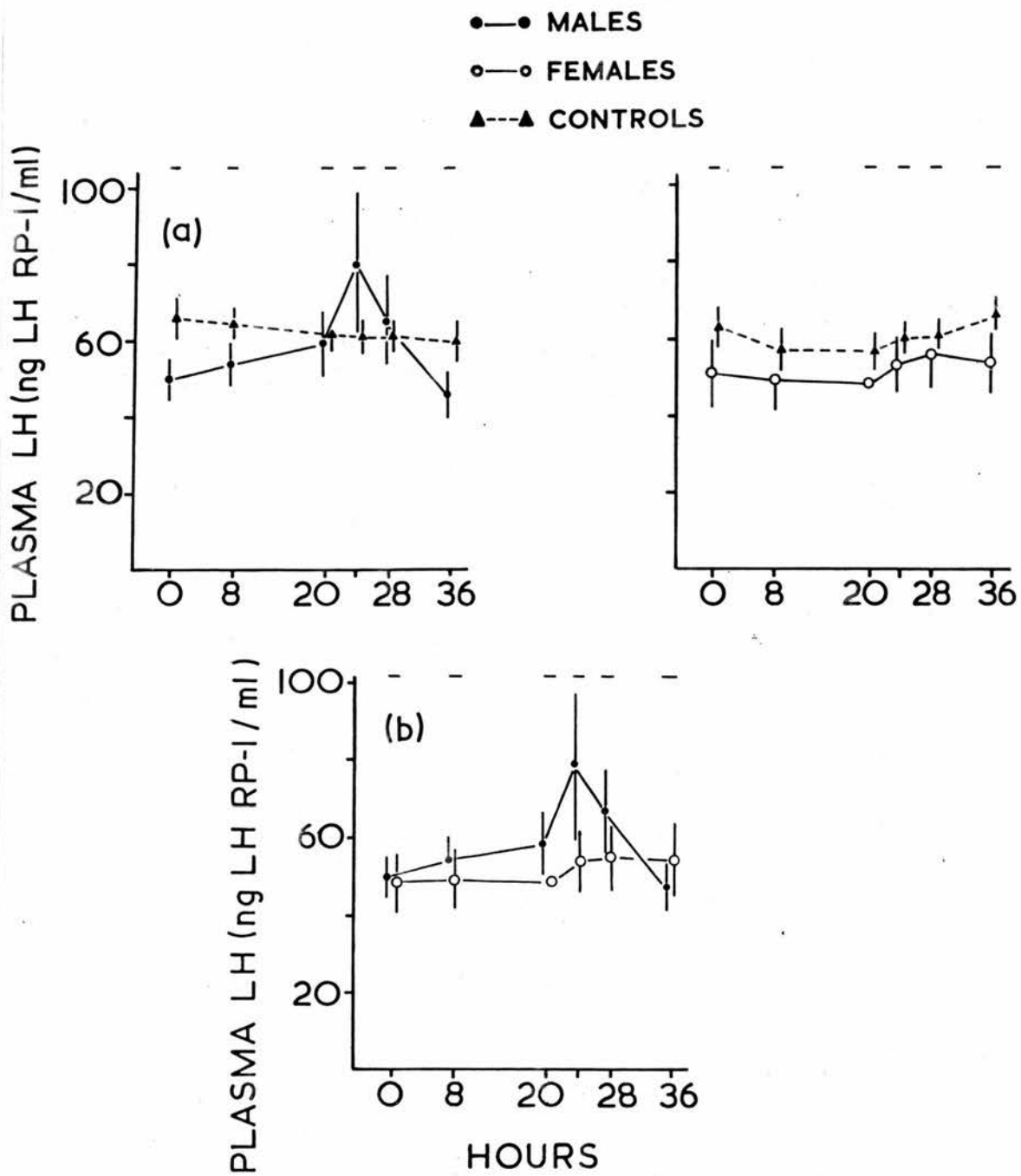


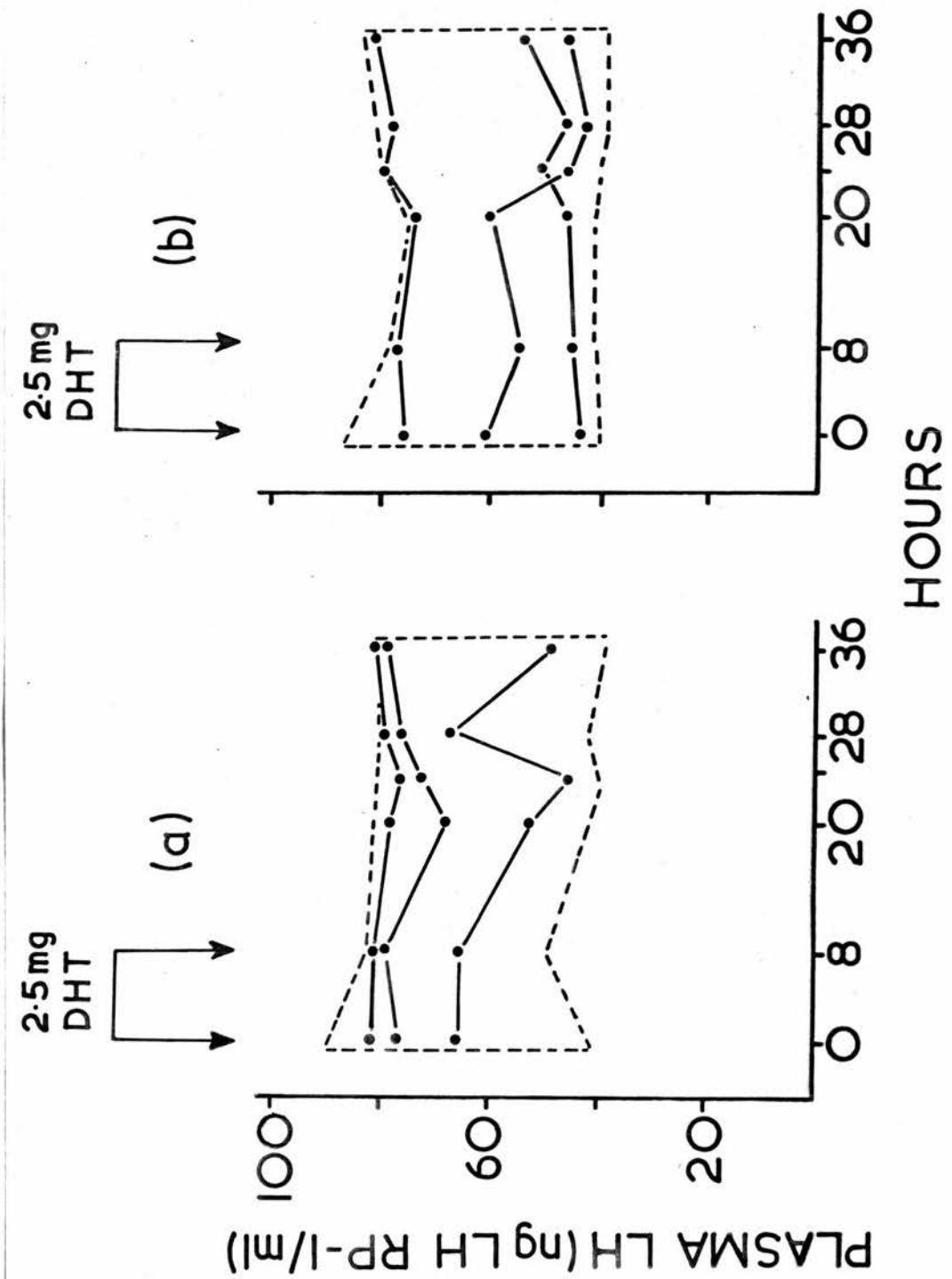
Figure 6.20

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=3) and females (n=3) and in respective controls (males and females, n=6).

Figure 6.21

LH concentrations in gonadectomised males (a) and females (b) after s.c. injections of 2.5mg DHT in oil at 0 and 8h.

Individual results for LH.



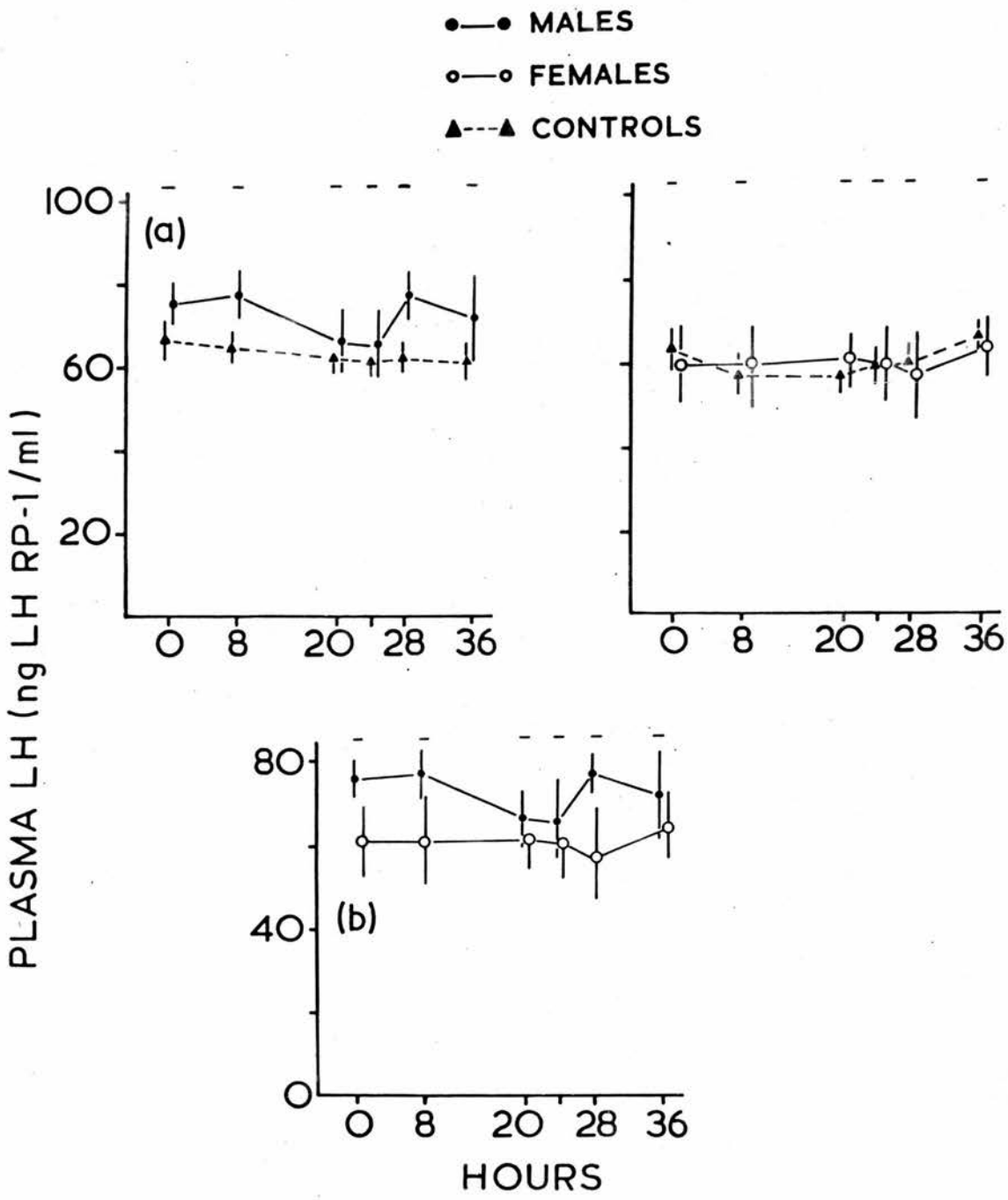


Figure 6.22

Mean (± S.E.M.) LH concentrations in gonadectomised males (n=3) and females (n=3) and respective controls (males and females, n=6)

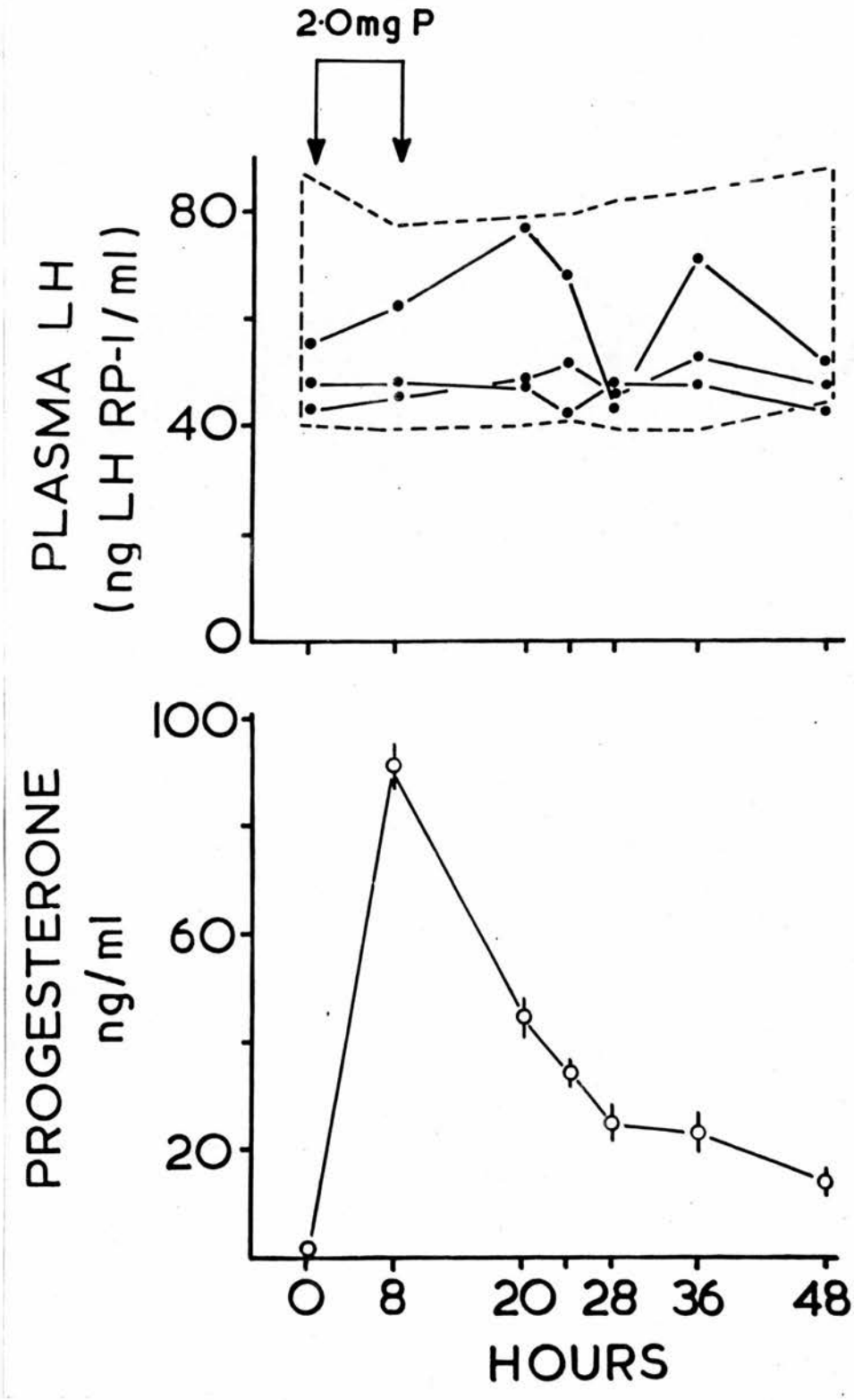


Figure 6.23

LH and progesterone concentrations in gonadectomised females after s.c. injections of 2.0mg P at 0 and 8h.

Individual results for LH; progesterone values are Mean ± S.E.M. (n=3).

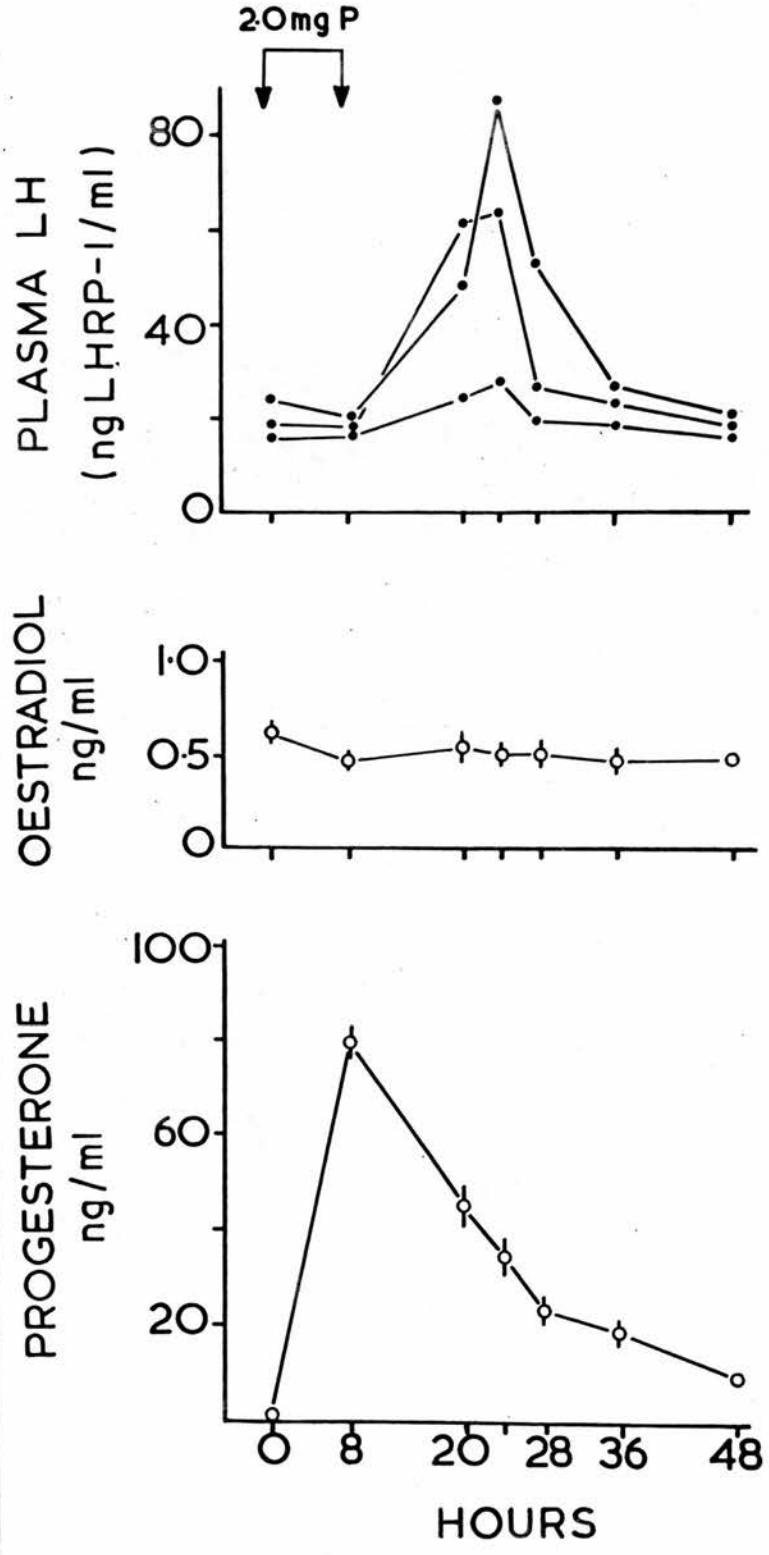


Figure 6.24

LH, oestradiol and progesterone concentrations in gonadectomised female marmosets after s.c. injections of 2.0mg P at 0 and 8h in animals containing oestradiol implants

Individual results for LH; oestradiol and progesterone values are Mean ± S.E.M. (n=3).

6.6 Discussion

A single injection of ODB had a biphasic effect on LH secretion in ovariectomised marmoset monkeys, with a negative feedback action characteristically preceeding the positive feedback effect. The initial suppression of LH secretion,which appears to occur as early as 4h after the administration of oestrogen,is followed by an abrupt increase in LH secretion, and maximum LH concentrations occur approximately 24h after the injection of ODB. A similar biphasic response to oestrogen is well documented in other primate species, although the LH surge occurs earlier in the marmoset than in the rhesus monkey and women, in which maximum LH concentrations are observed between 36 and 48h (Karsch, Dierschke, Weick, Yamaji, Hotchkiss and Knobil, 1973; Karsch et al, 1973a), and 72 and 96h (Yen and Tsai, 1972; Monroe et al, 1972) respectively after administration of oestrogen.

The dose of ODB administered produced a sustained rise in peripheral plasma oestradiol concentrations to give maximum levels similar to those (0.8 - 2.0 ng/ml) found in intact females immediately before the spontaneous LH surge in the normal cycle (Hearn and Lunn, 1975). Although oestradiol concentrations fell progressively after 8h, the observed positive discharges of LH cannot be associated with an "escape" from the negative feedback action of this hormone, since in general oestradiol levels remained in excess of 1.0 ng/ml until after the LH surge had occurred.

It can be seen from Figure 6.1 and 6.3 that positive and negative feedback was not observed in all the females tested. The failure of some animals to show negative feedback is probably due to the rather severe criteria which have been used in the definition of negative feedback. Thus LH levels in all treated animals were

lower after 8h compared with the pre-injection values, although in three cases these levels were less than two standard deviations below the mean control values. Failure to induce positive feedback in 100% of animals has also been reported in studies on rhesus monkeys (Yamaji et al, 1971; Karsch et al, 1973b) and as it does not, at least in the present study, appear to be associated with differences in circulating levels of oestradiol, individual variation in hypothalamic and/or pituitary thresholds of sensitivity to oestradiol may provide an explanation.

The pattern of LH release in gonadectomised male marmosets following an injection of ODB was broadly similar to that described for gonadectomised females. However, the LH response to ODB was more varied in intact males than in gonadectomised animals, and maximum concentrations of LH in the intact males which showed positive feedback were attained slightly later (28-36h) than in gonadectomised animals (24h). Nevertheless the mean concentrations of LH following ODB in the intact males tested were not significantly different from those observed in either male or female gonadectomised animals. The incidence of positive feedback in intact males ($^{9}/16$), however, was lower than in gonadectomised animals ($^{6}/8$ males; $^{7}/8$ females), although due to the small number of animals used it is difficult to know whether this represents a real difference. From the present data it is not possible to make a detailed quantitative comparison between male and female responses to ODB, for this would require a larger number of animals and the use of various doses of ODB. Although it is tempting to conclude that male marmosets show a female-type LH response to an oestrogen challenge, all that can be said is that male marmosets possess central nervous systems and/or pituitary mechanisms for the

release of LH after an oestrogen stimulus, and that the capacity to display positive feedback does not appear to be a sexually dimorphic characteristic in the marmoset monkey.

The fact that maximum levels of LH occurred later in intact males than in gonadectomised animals may indicate a difference in the type of response, suggesting a possible difference in sensitivity to the oestrogen stimulus. Since there was no difference in the time at which maximum LH concentrations were achieved between male and female gonadectomised animals, any differences in the response of intact males may be due to an action of testicular steroids. Thus testosterone and/or some other testicular product may influence hypothalamic and/or pituitary mechanisms thus causing a delay in the production of an LH discharge. Knobil (1974) has suggested that testosterone is responsible for abolishing the positive feedback response to oestrogen in intact male rhesus monkeys, and the release of LH in response to oestrogen, which has been observed in intact men (Dörner et al, 1975; Kulin and Reiter, 1976), was considerably diminished compared with that in women.

In the present study testosterone concentrations following an injection of ODB were markedly reduced during the initial stages of the LH response (Fig. 6.7). However, when elevated concentrations of testosterone were maintained for 20-24h after the administration of oestrogen to gonadectomised animals, positive feedback was not inhibited. It would therefore seem that the expression of positive feedback is not prevented by the presence of testosterone, and although in four animals maximum LH concentrations occurred after 28h (Fig. 6.10) the data are insufficient to conclude that elevated testosterone levels delayed the appearance of the LH surge. Exposure to

testosterone for several days before (as well as following) the injection of oestrogen (similar to the situation in intact males) may have a more marked effect on the pattern of LH release, although this remains to be determined.

The effect of dihydrotestosterone (a potent, non-aromatisable androgen) on oestrogen induced LH release was also tested and found to be ineffective in blocking the positive feedback response. Although circulating levels of dihydrotestosterone were not measured the dose administered was the same as for testosterone. It is therefore unlikely that the inability of dihydrotestosterone to block positive feedback was due to the dosage being inadequate to produce sufficiently elevated circulating levels, although the exposure time may not have been long enough.

Studies on the effect of progesterone on oestrogen induced LH release in rhesus monkeys and women have produced interesting, yet confusing, results. In the present study progesterone treatment (either with injections or implants) did not inhibit the initial negative feedback action of oestradiol on LH secretion. Whether progesterone synergises the negative feedback action of oestradiol, as suggested in ovariectomised rhesus monkeys (Dierschke et al, 1973), is not clear from these results in the marmoset. Simultaneous administration of oestrogen and progesterone in ovariectomised rhesus monkeys advances the time of the LH surge (Clifton et al, 1975), whereas in the present study progesterone did not have this effect. Although progesterone implants were used by Clifton et al (as opposed to injections in this study) circulating progesterone concentrations in both experiments were in the mid-range of luteal phase levels, and differences in the relative concentrations of progesterone is an

unlikely explanation for the different observations. Perhaps the relationship between progesterone and oestradiol levels is of importance, and it may be possible that the circulating levels of oestradiol achieved in the rhesus monkey were more susceptible to a synergistic action of progesterone.

As progesterone can inhibit oestrogen induced LH release in intact female rhesus monkeys (Spies and Niswender, 1972; Hess and Resko, 1973; Dierschke et al, 1973) and women (Netter et al, 1973), but not in ovariectomised rhesus monkeys (Clifton et al, 1975), it has been suggested that the ovary is necessary either directly or indirectly for the blocking effect of progesterone (Clifton et al, 1975). However this argument does not hold for the present data on the marmoset. Progesterone implants (achieving circulating concentrations similar to those obtained by injection) when maintained in position for 8 days before, and during the oestrogen provocation test, inhibited the positive feedback action of ODB. Two animals showed positive feedback, although maximum LH concentrations achieved were considerably lower than usual. Although it cannot be said that this regimen of progesterone treatment completely abolishes oestrogen induced positive feedback it is reasonable to conclude that it markedly reduces the positive response (mean LH concentrations 24, 28, 36h after the injection of oestrogen are significantly lower with this treatment ($p < 0.05$ students t-test), than those observed with oestrogen and progesterone injections (c.f. Figs. 6.16 and 6.18). The length of exposure of the hypothalamic-pituitary system to progesterone may therefore be of importance in determining its inhibitory influence on oestrogen induced positive feedback.

In addition to its inhibitory effect, progesterone may, under certain conditions have a stimulatory action on LH release. Thus progesterone can trigger an acute surge of LH after oestrogen priming in hypogonadal women (Odell and Swerdloff, 1968; Leyendecker et al, 1972) and castrated men (Stearns et al, 1973), and a similar effect has also been demonstrated in the late, but not in the early follicular phase in normally cycling women (Yen et al, 1975a). In the present study progesterone induced LH release in two of the three females with oestrogen implants, but no stimulatory effect could be observed in the females without oestrogen. Although inconclusive, the data on the marmoset tend to support the theory that oestrogen is necessary for progesterone to stimulate LH secretion. Since plasma progesterone concentrations are slightly elevated around the time of periovular LH release in rhesus monkeys (Weick, Dierschke, Karsch, Butler, Hotchkiss and Knobil, 1973) and women (Johansson and Wide, 1969), the possibility that progesterone may act synergistically with oestrogen must be considered.

The ability of testosterone to induce positive feedback has not previously been reported in primates. A single injection of testosterone can cause ovulation in anoestrous ewes (Radford and Wallace, 1971), and can elicit a similar LH response to oestradiol-17β in ovariectomised ewes (Clarke, 1976). At the dose rates employed in the present study, testosterone induced positive feedback in 2 out of 3 males, but surprisingly, it was ineffective in the females. Although not enough animals were tested in this study to say definitely that testosterone will not induce LH release in female marmosets, similar results have been obtained in gonadectomised rhesus monkeys (Martensz, pers. comm.). The reason for this apparent difference is, however,

obscure. Although a periovulatory rise in testosterone secretion has been observed in rhesus monkeys (Hess and Resko,1973), women (Vermeulen & Verdonck, 1976) and baboons (Goncharov et al, 1976) its significance is not yet clear, and it may be worth noting that in female rats, immunisation against testosterone did not affect pre-ovulatory LH release or ovulation (Gay and Tomacari, 1974). It is possible that testosterone may exert a specific effect on LH secretion per se, or by acting as a precursor for oestrogen synthesis in the brain (Naftolin et al, 1975, 1976). The fact that dihydrotestosterone, a non-aromatisable androgen, did not induce positive feedback in any of the marmosets tested may be interpreted to mean that the positive action of testosterone on LH release is mediated through oestrogens. Clarification of this point and the role of testosterone in controlling LH secretion in female primates must await the results of more detailed experiments. With respect to the observations described in Chapter 5, it is of interest to note that neither testosterone nor dihydrotestosterone had a negative feedback effect on LH secretion in this particular study.

In conclusion, the action of oestradiol-17β appears to be an important component of the positive feedback control of LH secretion in the marmoset. Furthermore, oestradiol-17β will induce a positive discharge of LH in males, as well as females. More detailed investigation is required to determine the physiological role of other steroids in regulating the pre-ovulatory LH surge.

6.7 Chapter Summary

1. A single injection of ODB, achieving circulating levels similar to those seen in intact females immediately before the mid-cycle LH

surge, had a biphasic effect on LH secretion in ovariectomised marmosets, with a negative feedback action characteristically preceeding the positive feedback effect. Under the experimental conditions, negative feedback could be seen by 4h after the injection, while maximum LH levels occurred after 24h.

2. Similar oestrogen treatment also induced negative followed by positive feedback in castrated and intact male marmosets, although maximum LH concentrations appear to occur slightly later in intact animals (after 28h) than in gonadectomised animals (after 24h).

3. Neither T nor DHT inhibited ODB induced LH release in gonadectomised animals, and the apparent delay in the appearance of peak LH levels in intact males could not, from the present results, be attributed to an action of these androgens.

4. Whereas injections of T induced LH release, albeit only in some males, no effect of DHT could be observed. This may indicate that the action of T was mediated through conversion to oestrogen, but clarification of this point is required.

5. Oestrogen induced positive feedback was apparently inhibited by long term prior exposure of the hypothalamic-pituitary system to luteal phase levels of P, but not when the hormone was injected simultaneously with the oestrogen.

6. P can induce LH release, but seemingly only when oestrogen is also present.

7. As in most of the experiments described in this study only a single dose and duration of steroid treatment was tested, interpretation of the results should be limited accordingly.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 General Discussion

The purpose of these studies was to investigate some of the mechanisms controlling LH secretion in the marmoset monkey. Since it is the first study in a new world primate, much of the work is preliminary and is limited to providing basic information for future and more rigorous investigation. However, the study was also designed to extend some previous observations made in the rhesus monkey and human, with an aim to furthering the understanding of the control of LH secretion in primates.

The results obtained demonstrate that synthetic LH-RH induces a marked increase in pituitary LH secretion and this, together with the data on inhibition of LH-RH, provides evidence that LH secretion in the marmoset is stimulated by LH-RH and that the releasing hormone has an important physiological role in the control of LH secretion in this species. The data also suggest that the endogenous releasing hormone in the marmoset closely resembles the decapeptide described by Matsuo *et al* (1971), and that the relative insensitivity of the rhesus monkey to the administration of synthetic LH-RH (Ehara *et al*, 1972; Arimura *et al*, 1973) cannot be adequately explained in terms of dissimilarities between the synthetic peptide and simian LH-RH.

Whilst LH secretion in the marmoset is directly related to LH-RH stimulation, the function of the hypothalamic-pituitary system appears to be profoundly modified by the action of gonadal steroids. This action, which forms the basis of feedback mechanisms, completes the hypothalamic-pituitary-gonadal system which regulates LH secretion in primates. In agreement with the well established theory of steroid feedback, both positive and negative feedback mechanisms operate in the marmoset.

An increase in circulating LH concentrations following gonadectomy provides clear evidence that LH secretion in the marmoset is normally suppressed by gonadal steroids (Hodges, 1978). That this negative feedback action is at least partially directed at the pituitary gland is suggested by an enhanced responsiveness to exogenous LH-RH in gonadectomised animals, which is probably due to an increase in pituitary LH content similar to that reported after castration in humans (Ryan 1962) and rats (Gay and Hauger, 1977). An increase in LH-RH secretion, as described in long term castrated rats (Seyler, Mitnick, Gordon and Reichlin, 1973) and men (Seyler and Reichlin, 1974) may also contribute to the increased LH secretion through a self-priming action on the pituitary, resulting in a large readily releasable pool of LH with a lower pituitary reserve (Lasley et al, 1975; Hoff et al, 1977).

The elevated LH concentrations in gonadectomised marmosets appear to be the resultant of rhythmic pulsatile discharges of the hormone by the pituitary gland. This so called episodic type of secretion, previously reported in various other species (see Chapter 1 for references) is most likely to reflect a pulsatile secretion of LH-RH from hypothalamic neurons. Thus, in the "open loop" situation pulsatile secretion of LH appears to reflect a basic functional rhythmicity in the hypothalamic-pituitary system. Whether this persists in the presence of steroid action, as in intact marmosets, remains to be determined.

Of the gonadal steroids involved in regulating LH secretion in the marmoset, oestradiol-17β appears to be particularly effective in both males and females. Since the enhanced pituitary responsiveness to LH-RH caused by gonadectomy can be functionally reversed by

oestradiol alone, with a resulting suppression of LH secretion, it is apparent that oestradiol is involved in the negative feedback control of LH secretion in the marmoset, and a direct inhibitory action on the pituitary indeed represents an important component of this control mechanism. While it is clear that the chronic effects of oestradiol-17β are inhibitory, the shorter term stimulation of LH release described in Chapter 4 is consistent with observations in rhesus monkeys (Knobil, 1974) and humans (Yen et al, 1975a), that oestrogen induced effects on LH secretion are time and dose related. In this respect it is worth reiterating that attempts to correlate the feedback effects of oestradiol, or indeed any other steroid, with changes in pituitary responsiveness to LH-RH are also complicated by the apparent existence of two functionally separable pools of pituitary LH (Wang et al, 1976 ; Hoff et al, 1977). The mode of administration of LH-RH is clearly important in obtaining an accurate reflection of pituitary function, and this should be considered in any subsequent studies on pituitary response to LH-RH in the marmoset. Thus a procedure which would discriminate between pituitary sensitivity and reserve should, if possible, be adopted. As few as two injections of a sub-maximal dose of LH-RH (e.g. 0.2 ug) 90 min apart should, with carefully timed blood sampling, be sufficient to gain considerable additional information on steroid induced changes in pituitary response to LH-RH.

Definition of the feedback roles of other steroids in controlling tonic LH secretion in the marmoset is not possible from the present, rather preliminary data, and must await a more thorough investigation. In addition to dose and duration of treatment, an important consideration when testing the feedback properties of

these steroids appears to be the interval between castration and the initiation of treatment. The results in the marmoset confirm previous observations in rats (Swerdloff and Walsh, 1973; Legan and Karsch, 1975; Zanisi and Martini, 1975), sheep (Brown et al, 1972; Karsch et al, 1976) and rhesus monkeys (Resko et al, 1977), that the inhibitory effects of steroid hormones (including oestradiol, progesterone and testosterone) on gonadotrophin secretion decrease as the post-castration time increases. However, as yet there has been no adequate explanation to account for these apparent changes in feedback sensitivity. One possible explanation is that in the absence of normal levels of circulating steroids, there is a gradual depletion in the number of specific receptors for these steroids in the pituitary and/or hypothalamus. It would thus be expected that an increase in the interval from castration will be associated with a progressive decline in the receptor population. A reduction in circulating steroid hormone binding globulin after castration may also be a possibility. Seyler and Reichlin (1974b) have reported that although castration in men results in an increase in LH-RH secretion, the increase could not be detected until 30 days after castration. These observations, if correct, may be extended to other species and the possibility that the relative ineffectiveness of steroids to inhibit LH release in long term castrates may be related to the delayed increase in LH-RH secretion is worth consideration. It would be interesting to see whether implants of the steroids used in this study, in physiological doses which are effective in preventing the post castration LH rise, would maintain low levels of LH over an extended period, or whether a change in the sensitivity of the hypothalamic-pituitary axis to negative feedback would still occur, with a resulting gradual rise in LH concentration, despite a constant level of circulating steroid.

From the present results it is reasonable to assume that oestradiol-17β plays a major role in the feedback control of tonic LH secretion in female marmosets. The significance of the apparent negative feedback action of progesterone in the marmoset is not clear, and there is not yet sufficient information to define the physiological role (if any) of progesterone in regulating tonic LH secretion in this species. If progesterone is of importance, it may be assumed that since the progesterone treatment used in this study enhanced pituitary responsiveness to LH-RH, any inhibition of LH secretion is likely to involve an action at the hypothalamic level.

Although oestradiol-17β can also inhibit LH secretion in male marmosets, the physiological significance of testicular oestrogen is not fully understood. A combined action of androgens and oestrogens is more likely to reflect the feedback control of LH secretion in males, rather than any individual action, and in this respect Resko et al (1977) have demonstrated a synergistic role of testosterone and oestradiol in male rhesus monkeys. Testosterone can reduce pituitary responsiveness to LH-RH in male rats (Debeljuk, Arimura and Schally, 1972a; Cheung and Davidson, 1977) and men (Muhlen and Kobberling, 1973), and the effects of testosterone and other androgens on LH-RH induced LH release in the marmoset should also be investigated. This may provide useful information on the feedback roles of androgens, and would in fact be a practical method for testing negative feedback in intact marmosets.

It is well established that for a variety of species (see Chapter 1) the pre-ovulatory rise in circulating oestradiol-17β represents the critical stimulus for the initiation of the mid-cycle

LH surge. Oestradiol-17β will also induce a positive discharge of LH in female marmosets, and it is therefore likely that oestradiol-17β plays an important role in initiating the pre-ovulatory LH surge in this species. This stimulatory or positive feedback action of oestradiol on LH secretion was followed (Chapter 5) and invariably preceded (Chapter 6) by a decline in circulating LH attributable to the negative feedback action of the steroid. In agreement with observations in the rhesus monkey (Knobil, 1974) and women (Yen et al, 1975a),the positive feedback effect of oestradiol-17β in the marmoset is not simply a function of some threshold plasma concentration of the steroid, but must also be critically dependent on a time component. At present however, neither the precise duration of the effective stimulus nor its threshold level have been determined.

The precise mechanisms involved in controlling the midcycle LH surge in the marmoset, as well as in other species, remain unclear, and in particular the roles of progesterone and androgens have not been adequately explained. Under the experimental conditions described in Chapter 6, testosterone and dihydrotestosterone neither inhibited nor enhanced oestrogen induced positive feedback. A midcycle elevation in testosterone, dihydrotestosterone and androstenedione has been reported in certain primates (see Chapter 1 for references),and it would seem unlikely that these androgens are physiologically totally inert with respect to the midcycle gonadotrophin surge. Thus they may have a permissive role, and in this regard Martensz et al (1976) have reported that elimination of androstenedione by immunisation inhibited oestrogen induced LH release in ewes.

The role of progesterone in the pre-ovulatory LH surge remains an enigma. In agreement with previous observations in other species,

the present data confirm that under the appropriate experimental conditions, progesterone can either induce positive feedback (Odell and Swerdloff, 1968; Yen et al, 1975a), or inhibit oestrogen induced positive feedback (Netter et al, 1973; Dierschke et al, 1973). Furthermore, from the results in the marmoset, it would appear that whereas long term previous exposure of the hypothalamic-pituitary system to physiological levels of progesterone inhibits oestrogen induced LH release, progesterone, when administered simultaneously with oestrogen, shows no apparent inhibition. Although these observations are consistent with the ideas formulated from other studies that luteal phase progesterone blocks oestrogen induced positive feedback (Dierschke et al, 1973), whereas peri-ovulatory progesterone may actually enhance the later stages of the mid cycle surge (Johansson and Wide, 1969; Leyendecker, Wardlaw and Nocke, 1972), results obtained in gonadectomised animals may not be entirely relevant to the situation during the cycle, and should therefore be viewed with caution.

Positive feedback was for a long time considered to be exclusively a female characteristic, although the adaptive significance of this is not obvious. Positive feedback is necessary for ovulation to occur, but the persistence of the response in the male would not interfere with normal testicular function. In recent years it has been suggested that the ability of the hypothalamic-pituitary unit to discharge LH in response to oestrogen may not be a sexually dimorphic characteristic in primates, as it is in rats and sheep. The present data confirm this and provide the clearest demonstration to date of a positive feedback effect of oestradiol-17β on LH secretion in a male primate (Hodges and Hearn, 1978). Although the data from the rhesus

monkey and men are not as convincing, there are indications that with respect to oestrogen induced LH release in males, primates may in fact differ from rats and sheep.

One possible explanation for this difference may arise from the fact that in primates, unlike rats and sheep, exposure to androgen during the critical period of development does not necessarily affect normal ovarian cyclicity during later life (see Chapter 1 for references). Several studies have suggested that when rats are exposed to testosterone during the critical stage of neural differentiation, the region of the brain which is rendered inoperative is the pre-optic area (POA) (Barraclough, 1966; Gorski, 1971; Libertun, Timiras and Kragt, 1973), and it is well established (Schwartz, 1969; Norman, Blake and Sawyer, 1973) that in rodents the initiation of the pre-ovulatory gonadotrophin surge is dependent on a signal generated by the POA of the brain. Thus surgical isolation of the POA from the medial basal hypothalamus (MBH) blocks the cyclic surge of gonadotrophin secretion and ovulation in the rat (Halasz and Gorski, 1967; Blake, Weiner, Gorski and Sawyer, 1972). In contrast, the work of Krey et al (1975) has shown that deafferentation of the MBH in the rhesus monkey does not have this effect, and they suggest that intact connections from the POA to the MBH are not required for the expression of the positive feedback action of oestrogen on LH and FSH secretion in this species. If therefore the POA is also the target for androgen action during the critical period in the rhesus monkey and other primates, then the ability to respond to positive feedback would not necessarily be abolished.

Furthermore, Knobil (1974) has postulated that in primates, the "clock" or "Zeitgeber" which determines the timing of ovulation

is not resident in the brain, as it appears to be in the rat (Everett 1961, 1964), but lies in the ovary. Thus in primates, where environmental stimuli (directed at the brain) appear to have become less important in controlling reproduction than in species like rats and sheep, functional differentiation between "male" and "female" at the neural level may not be so essential.

Whether or not the centres regulating positive feedback in the male have developed differently in all primates than in rats and sheep, it appears that oestrogen induced LH release in male marmosets is much more pronounced than in male rhesus monkeys or men. Although there is no obvious reason for this, the distinctive embryology of the marmoset may help to provide a partial explanation. Marmosets are unique among primates because of their high incidence of twinning and the presence of placental vascular anastomoses that occur between the twin foetuses (Wislocki, 1939; Gengozian, 1971). Furthermore, although this situation invariably leads to haemopoietic chimaerism, there is no evidence of freemartinism (Benirschke and Brownhill, 1962). Thus a rather special arrangement exists in the marmoset which allows normal and independent development of male and female co-twins despite their intimate relationship afforded by a common blood supply. Whether this unique relationship <u>in utero</u> has any direct relevance to the persistence of a positive feedback mechanism in the male, however, remains to be determined.

Although the intact male marmoset possesses the potential to display positive feedback, it would seem doubtful that this potential is ever realised during the normal course of LH secretion. Since ovarian function is almost certainly necessary to activate the positive feedback mechanism, if the practical problems could be

overcome, it would be interesting to see whether a cyclic pattern of LH secretion could be obtained in a castrated male marmoset implanted with ovaries.

Although there are many aspects of the control of LH secretion in the marmoset still to be investigated, the present study establishes the importance of the hypothalamic-hypophysial-gonadal system in this species, and describes some of the ways in which the various components of this system interact. The marmoset is the first new world primate to be subjected to detailed endocrinological investigation, and an understanding of its reproduction is therefore of certain academic interest alone. In addition, the similarities in the control of LH secretion which exist between the marmoset and other primates may allow an application of studies in the marmoset to certain aspects of reproduction in the human. One such application may lie in the development of new methods of human fertility control.

The results described in Chapter 3 indicate that active immunisation against LH-RH is an effective way of suppressing gonadal activity in the marmoset (Hodges and Hearn, 1977). The persistence of a certain amount of gonadal function, particularly in the animals with low antibody titres, would suggest that gonadotrophin secretion was not completely abolished, although data on the degree of inhibition of LH and FSH secretion in the marmoset are not available to confirm this. Clearly this requires further investigation and when an assay for measuring marmoset FSH becomes available, useful information on the effects of LH-RH immunisation on both LH and FSH secretion may be obtained from studies on castrated animals.

The apparent lack of species specificity of LH-RH and the observed breakdown of the hypothalamic-pituitary-gonadal axis

described in this study by immunisation against LH-RH may provide a method by which this can be achieved in several species, being particularly useful when hypophysectomy or gonadectomy are undesirable. Thus in addition to potential application as a form of contraception, inhibition of LH-RH may be useful in the veterinary field, and clinically, in the treatment of certain forms of breast cancer, precocious puberty and excessive and frequent menstruation towards the end of reproductive life.

However, for any practical application of LH-RH inhibition to be acceptable, particularly in fertility control, reversibility of the effects would be essential. In the present study natural reversal seems to occur following the decline of antibody titres, but as the data are incomplete a much more rigorous investigation is required to determine whether completely normal gonadal function is restored following a period of prolonged suppression. Although gonadotrophin secretion has been induced in immunised rats with immunologically cross reactive fragments of LH-RH (Fraser, 1977) and with super active LH-RH analogues (Fraser and Sandow, 1977), it is not yet known how the gonads respond to the gonadotrophic stimulus.

One further consideration which at present precludes the acceptability of immunisation against LH-RH as a method of fertility control is the necessity for adjuvants. Freunds complete adjuvant is most effective (Fraser, 1976b) but cannot be used in human or vetinerary work. A dilemma exists in that the properties which make a good adjuvant also contribute to side effects. In the marmoset, the use of Freunds adjuvant induced the formation of lesions at the immunisation sites, and clearly other effective but harmless adjuvants need to be developed.

In view of the rather drastic effects of immunisation against LH-RH, antagonistic analogues of LH-RH have recently attracted considerable attention as an alternative form of LH-RH inhibition. The two analogues tested in the present study both suppress LH-RH induced LH release, but the rapid degradation of these substances *in vivo* reduces their effectiveness. However, repeated injections of these peptides can block ovulation in the rat (de la Cruz *et al*, 1976; A. V. Schally, *pers comm*), and with the synthesis of much longer acting compounds which is already in progress, use in humans may be feasible.

Thus it may still be possible to develop a new approach to contraception by LH-RH inhibition, achieved either immunologically or by the use of competitive LH-RH antagonists. Total suppression of the testis would be necessary in the male to ensure infertility, and this would probably be undesirable. In the female, however, it may be possible to suppress the ovary to a level which would effectively prevent ovulation, but would still allow some follicular development and oestrogen secretion.

7.2 Conclusion

LH-RH has an important physiological role in controlling LH secretion in the marmoset monkey, although the relationship between the hypothalamus and the pituitary gland is profoundly influenced by the feedback actions of steroid hormones. Thus LH secretion is normally suppressed by gonadal steroids which act, at least in part, directly on the pituitary gland. Oestradiol-17β probably represents a major component of this negative feedback mechanism in females, and may also be important in males, where presumably it acts in association with androgens. Although progesterone has been shown to inhibit LH

secretion, the physiological significance of this action remains to be determined. By means of a separate feedback mechanism oestradiol-17β will also induce a positive discharge of LH. This action of oestradiol-17β is almost certainly responsible for initiating the pre-ovulatory LH surge in females, although in the normal cycle, other steroids may be important in modulating this positive feedback action. The persistence of a positive response to oestrogen in males suggests that in the marmoset, the ability to discharge LH in response to oestrogen is not a sexually dimorphic characteristic, although the involvement of the response in the control of LH secretion in males is unlikely.

The hypothalamic-hypophysial-gonadal system, responsible for controlling LH secretion in the marmoset can be disrupted by inhibition of LH-RH action, achieved either immunologically by active immunisation, or by the use of competitive antagonistic analogues of LH-RH. These procedures may find an application in vetinerary and clinical treatment of reproductive disorders, and may eventually provide a new approach to contraception in the human.

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