Control of Inhibin Production and Secretion in the Primate Ovary

Karen Brooks Smith B.Sc. (Hons)

Doctor of Philosophy
University of Edinburgh
January 1991

For Mum, Dad, Raymond and Neil.

Table of Contents

Page

Page

List of Figures and Tables

Page

Page

List of Plates

Acknowledgements

I am grateful to Dr. Hamish Fraser for his excellent supervision and guidance throughout this project, which was funded by the Medical Research Council. I wish to thank Dr. Stephen Lunn for his many words of wisdom (and fun!) and photographic expertise and Mrs. Gwen Cowen for teaching me practical and organizational skills in the laboratory. I am grateful to Mr. Mike Millar for teaching me the basics of histology and for working with me to set up immunocytochemistry.

I wish to thank Professor David Baird for allowing me to collaborate in the clinical studies and for his encouragement throughout this project. I also wish to thank Dr. Peter Illingworth for working with me to establish the luteal cell culture system, for organizing the collection of tissue and for his knowledge of who did what, when and in what journal! I am grateful to Dr. Kogie Reddi and Mrs Sodsai Tovanabutra for sharing their knowledge and experience of laboratory techniques with me, especially with the inhibin assay, and for their friendship.

I also wish to thank Dr. Alan McNeilly for his enthusiasm and encouragement. I am grateful to Mr. Tom McFetters and Mr. Ted Pinner for their artistic skills over the past three years, for advice on the preparation of this thesis and for making me laugh. I am grateful to Mrs. Margaret Harper for her excellent secretarial skills and especially for her help in fund raising for my trip to California. I also wish to thank Ms. Erin McNeill, Mr. Duncan Porter, Ms. Julie Griffiths and Ms. Katrina Tainsh for their friendship and encouragement, especially during the 'writing-up' stage.

And finally, I wish to thank Mum and Dad, my brother Raymond and my fiancé Neil. This Ph.D. would not have been possible without their constant moral and financial support throughout my time as a student and this thesis is dedicated to them.

Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Karen Brooks Smith

January 1991

Abstract

The studies described in this thesis were designed to investigate the control of inhibin production, secretion and localization in the primate ovary. A heterologous radioimmunoassay was established and validated for the measurement of inhibin during the normal menstrual cycle in the stumptailed macaque (*Macaca arctoides*) and ovulatory cycle in the common marmoset monkey (*Callithrix jacchus*). The pattern of immunoreactive inhibin secretion was low during the follicular phase, reaching maximum levels during the mid-luteal phase in both species. This is similar to the pattern observed in the human. These results suggest that the corpus luteum is a major source of immunoreactive inhibin secretion in the primate. Inhibin concentrations remained elevated in pregnant marmosets throughout gestation.

The gonadotrophic control of inhibin production was investigated *in vivo* by administration of a luteinizing hormone releasing-hormone (LHRH) antagonist in the stumptailed macaque during the mid-luteal phase. Treatment with LHRH antagonist for 3 days resulted in permanent suppression of luteal function as shown by low serum concentrations of progesterone and immunoreactive inhibin. Replacement of gonadotrophin with human chorionic gonadotrophin (hCG) but not follicle-stimulating hormone (FSH) prevented gonadotrophin induced suppression by antagonist suggesting that inhibin, similar to progesterone, is integrated with the luteinizing hormone (LH) control of the corpus luteum. The control of inhibin secretion was further investigated in an *in vitro* luteal cell culture system. Human luteal cells secreted progesterone, oestradiol and inhibin in culture. Inhibin secretion by the luteal cells was stimulated by hCG in a dose-dependent manner providing further evidence that the secretion of inhibin is under the control of LH.

In an attempt to obtain a model of transitory suppression of luteal function, the effect of treatment with LHRH antagonist for 1 or 2 days during the mid-luteal phase on serum concentrations of progesterone and inhibin was compared. Recovery of progesterone and inhibin secretion was observed in two out of six macaques treated with two injections of antagonist and in three out of six treated with a single injection of antagonist. Therefore, with the regimens of LHRH antagonist employed, this approach was not conducive to obtaining a reliable transitory suppression of luteal function. The effect of ovarian hyperstimulation with FSH on serum concentrations of immunoreactive inhibin in stumptailed macaques in which endogenous gonadotrophin secretion and ovarian activity had been suppressed by an LHRH agonist implant was

studied. LHRH agonist treatment suppressed both steroids and inhibin. Administration of FSH for 9 days, 8 weeks after agonist implant, resulted in marked elevations in oestradiol and immunoreactive inhibin. This nonphysiological situation demonstrated that developing follicles may be a source of inhibin. However it requires the growth of multiple antral follicles to induce a marked rise in immunoreactive inhibin during follicular development.

Inhibin was localized immunocytochemically in the primate ovary using an avidin-biotin immunoperoxidase technique. Intense immunostaining for inhibin α- and β-subunits was detected within the granulosa-lutein cells of the human corpus luteum. Similar distribution of inhibin α-subunit immunostaining was observed in 12 corpora lutea obtained during early-, mid- and late-luteal phases of the menstrual cycle and no changes in intensity or distribution of staining were apparent at these different stages. The specific localization of inhibin within the granulosa-lutein cells suggests that inhibin production may originate from a discrete cell population within the corpus luteum.

Abbreviations

AEC	3-amino-9-ethylcarbazole
cAMP	cyclic adenosine monophosphate
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	diacylglycerol
DARS	donkey anti-rabbit serum
$DHKF_{2\alpha}$	13,14-dihydro-15-keto-$PGF_{2\alpha}$
FSH	follicle stimulating hormone
FSP	FSH-suppressing protein (follistatin)
GAP	gonadotrophin hormone releasing- hormone associated peptide
hCG	human chorionic gonadotrophin
HPLC	high pressure liquid chromatography
IP_3	inositol-1,4,5-triphosphate
IVF	*in vitro* fertilization
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
NRS	normal rabbit serum
oRTF	ovine rete testis fluid
PAP	peroxidase anti-peroxidase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
pmp	post-menopausal plasma
pphff	partially purified human follicular fluid
QC	quality control
RIA	radioimmunoassay
TGF-β	transforming growth factor β

Publications

1. Smith, K.B., Lunn, S.F., Fraser, H.M. (1990)
' Inhibin secretion in the common marmoset monkey.'
Journal of Endocrinology 126, 489-495.

2. Smith, K.B., Fraser, H.M. (1991). 'Control of progesterone and inhibin secretion during the luteal phase in the macaque.'
Journal of Endocrinology 128, 107-113.

3. Smith, K.B., Millar, M.R., McNeilly, A.S., Illingworth, P.J., Fraser, H.M., Baird, D.T. (1991). Immunocytochemical localisation of inhibin in the human corpus luteum. Journal of Endocrinology 128.

4. Smith, K.B., Fraser, H.M., Reddi, K., Robertson, D.M., de Kretser, D.M.(1989). 'Gonadotrophic control of inhibin concentrations in serum during the luteal phase in the macaque.'
Journal of Reproduction and Fertility, Abstract Series No.3 July 1989, Abstract no.62.

5. Smith, K.B., Fraser, H.M.(1989)
'Inhibin secretion in the female macaque: manipulation by LHRH analogs and exogenous gonadotropins.'
Abstract 32 in 'Neuroendocrine Regulation of Reproduction,' Proceedings of Serono Symposium, Napa Valley, U.S.A. Nov 1989

6. Smith, K.B., Lunn, S.F., Fraser, H.M. (1990).
' Inhibin secretion in the common marmoset monkey.'
Journal of Endocrinology, 124 (suppl) Abstr.249

7. Smith, K.B., Millar, M.R., McNeilly, A.S., Illingworth, P.J., Fraser, H.M., Baird, D.T. (1990)
' Immunocytochemical localisation of inhibin in the human corpus luteum.'
Journal of Reproduction and Fertility, Abstract Series No.5 July 1990, abstract 64.

8. Smith, K.B., Fraser, H.M., Reddi, K., Robertson, D.M., de Kretser, D.M.(1989). 'Gonadotrophic control of serum inhibin during the luteal phase in the macaque.'
In'Simpson Symposium Number 2-1989; Maternal and Fetal Medicine.' p 51.

9. Fraser, H.M., Smith, K.B., Reddi, K., Lunn, S.F.(1990)
'Ovarian hyperstimulation induced by FSH causes supraphysiological secretion of inhibin during the follucular phase in the macaque.'
Journal of Reproduction and Fertility 89, 441-446.

10. Illingworth, P.J., Reddi, K., Smith, K.B., Baird, D.T.(1990)
'Pharmacologic "Rescue" of the Corpus Luteum results in increased Inhibin Production.'
Clinical Endocrinology 33, 323-332

11. Illingworth, P.J., Reddi, K., Smith, K.B., Baird, D.T. (1991)
' The source of inhibin secretion during the human menstrual cycle.'
accepted for publication, Journal of Clinical Endocrinology and Metabolism 127 (3).

12. Illingworth, P.J., Reddi, K., Smith, K., Baird, D.T.(1989)
' Ovarian vein concentrations of Inhibin during the human menstrual cycle.'
Journal of Endocrinology,123(suppl) Nov.1989, Abstract 62.

13. Illingworth, P.J., Reddi, K., Smith, K.B., Baird, D.T.(1989)
'Pharmacologic "rescue" of the corpus luteum results in increased inhibin production.'
In 'Simpson Symposium Number 2-1989; Maternal and Fetal Medicine.'p. 46.

14. Illingworth, P.J., Reddi, K., Smith, K.B., Baird, D.T.(1990)
' The effects of endogenous LH pulsatility and exogenous hCG administration on the peripheral concentration of inhibin.'
In 72nd Annual Meeting of American Endocrine Society Meeting, 1990, abstract 1378.

15. Baird, D.T., Fraser, H.M., Hillier, S.G., Illingworth, P.J., McNeilly, A.S., Reddi, K., Smith, K. (1989)
'Regulation of Ovarian Inhibin Production and Secretion in Women.'
In 'Neuroendocrine Regulation of Reproduction,' Proceedings of Serono Symposium, Napa Valley, U.S.A. Nov 1989 pp195-205.

16. Reddi, K., Illingworth, P.J., Saunders, P.T.K., Smith, K.B., Hillier, S.G., Baird, D.T.(1990)
' Expression of Inhibin Alpha Subunit mRNA in the Human Corpus Luteum is maintained throughout the Luteal Phase."
Journal of Endocrinology, 124 (suppl) Abstr.75

17. McNeilly, A.S., Reddi, K., Illingworth, P.J., Smith, K.B., Fraser, H.M., Tovanabutra, S. & Baird, D.T. (1990). Inhibin. In Simpson Symposia Number 3, Fertility Control and Reproductive Health in the 1990's.p. 28.

Presentations at conferences

1. Society for the Study of Fertility - Annual Conference, Canterbury, July 1989. Oral presentation - ' Gonadotrophic control of Inhibin concentrations in serum during the luteal phase in the macaque.' Smith, K.B., Fraser, H.M., Reddi, K., Robertson, D.M., de Kretser, D.M.

2. Simpson Symposium, Edinburgh, September 1989.
   Poster presentation - ' Gonadotrophic control of serum inhibin during the luteal phase in the macaque.' Smith, K.B., Fraser, H.M., Reddi, K., Robertson, D.M., de Kretser, D.M.

3. Serono Symposium ' Neuroendocrine Regulation of Reproduction ', Napa Valley, California, U.S.A. November, 1989.
   Poster presentation - ' Inhibin secretion in the female macaque: manipulation by LHRH analogs and exogenous gonadotropins.' Smith, K.B., Fraser, H.M.

4. British Endocrine Society - Annual Conference, Glasgow, March 1990.
   Poster presentation - ' Inhibin secretion in the common marmoset monkey ' Smith, K.B., Lunn, S.F., Fraser, H.M.

5. Society for the Study of Fertility- Annual Conference, Sheffield, July,1990. Oral presentation- 'Immunolocalization of inhibin in the human corpus luteum.' Smith, K.B., Millar, M.R., McNeilly, A.S., Illingworth, P.J., Fraser, H.M., Baird, D.T.

Chapter 1- Literature Review

Introduction

The control of female reproduction in human and non-human primates is the result of a complex series of hormonal interactions between the hypothalamus, pituitary and ovary. The aim of this review is to discuss this neuroendocrine axis and the physiological and pharmacological models which have been used to elucidate the feedback control mechanisms involved. In particular, this review will focus on the luteal phase of the primate menstrual cycle, the endocrine role of inhibin and the gonadotrophic control of inhibin by the primate corpus luteum.

1. Hypothalamic control of gonadotrophin secretion

The hypothalamic-pituitary-ovarian axis in primates is primarily under the control of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus. This decapeptide acts on the pituitary to promote the synthesis and release of the two major gonadotrophic hormones involved in the regulation of reproduction, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are composed of a common α-subunit and a β-subunit which confers specificity. These subunits are encoded by single genes which are regulated separately (Lalloz et al., 1988). Some studies on purification of hypothalamic extracts have suggested that FSH is controlled by a separate releasing hormone (McCann et al., 1986). However, after two decades of speculation an FSH-releasing hormone remains to be chemically identified and the consensus of opinion is that LHRH also controls FSH secretion. For the purpose of this thesis, the hormone responsible for the release of both gonadotrophins will be designated as LHRH.

1.1 Structure of LHRH

LHRH is produced from the post-translational processing of a larger molecule (Seeburg & Adelman, 1984; Seeburg et al., 1987). The precursor consists of a signal sequence, the ten amino acid LHRH sequence, a site for enzymatic processing and a 56 amino acid-long peptide which has been termed gonadotrophin hormone releasing hormone-associated peptide (GAP) (Seeburg & Adelman, 1984). The structure of LHRH is identical in all mammalian species and the amino acid composition of GAP is conserved about 85% between human, rat and mouse (Seeburg et al., 1987).

1.2 Localisation of LHRH

LHRH is synthesized in hypothalamic neurosecretory cells and is transported along axons and stored in the nerve terminals of the median eminence. Co-localization of LHRH and GAP within the cell bodies and terminals of this area was demonstrated immunocytochemically in the rat (Phillips et al., 1985) and primate (Song et al., 1986) brain. Furthermore, Ronnekleiv et al. (1987) showed that LHRH decapeptide was present within the cell bodies, nerve fibres and nerve terminals of the macaque and baboon pre-optic basal hypothalamus, whereas the precursor (proLHRH) was present only in the cells bodies. These results demonstrated that processing events within the LHRH neurons occur primarily in the cell soma and the cleavage products are then transported to the nerve terminals.

1.3 Secretion of LHRH

The central component governing the release of LHRH is a 'pulse generator' which is located in the region of the arcuate nucleus of the mediobasal hypothalamus (MBH) (Goodman & Karsch, 1981; Knobil, 1981). This pulse generator initiates a cascade of events beginning with the release of a bolus of LHRH into the pituitary portal system (Sherwood et al., 1976) approximately once every hour and transported down the infundibulum to the anterior pituitary gonadotrophs (for review see Sawyer, 1978). The study of the normal physiology of LHRH has been complicated by difficulties in the measurement of LHRH. The vast majority of LHRH secreted from the hypothalamus is predominantly confined to the portal blood supply linking the central nervous system to the anterior pituitary. As a result, levels of LHRH in the periphery are very low and its measurement is further confounded by its rapid half-life of 2-4 min (Arimura et al., 1974). Consequently, the study of the physiology of LHRH secretion involves a series of inferential approaches which must be taken into account when results are interpreted.

1.4 Pulsatility of LHRH

The evidence to support the pulsatile discharge of LHRH was obtained from 3 sources:

(i) LHRH was measured in blood samples collected from the pituitary stalk portal system of anaesthetized ovariectomized rhesus monkeys (Carmel et al., 1976) and rats (Sarkar & Fink, 1980; Soper & Weick, 1980).

(ii) Immunoneutralization of LHRH abolished the pulsatile secretion of LH in the ewe (Clarke et al., 1978; McNeilly et al., 1984), ram (Lincoln & Fraser, 1979), ovariectomized female rats (Snabels & Kelch, 1979) and stumptailed macaque (Fraser

et al., 1982; Fraser, 1986). In the female rat and sheep, but not stumptailed macque, this treatment inhibited the naturally occurring or steroid-induced preovulatory LH surge (Koch et al., 1973; Fraser, 1986; Fraser & McNeilly, 1982; 1983).

(iii) In the 'hypothalamic clamp' experiments of Knobil (1980), radiofrequency lesions were placed in the medial basal hypothalamus of ovariectomized rhesus monkeys. This promptly reduced plasma LH and FSH concentrations to undetectable levels. Using this model it was demonstrated that replacement of LHRH by short regular pulses restored normal gonadotrophin secretory profiles and induced regular menstrual cycles. Secondly, they established that the 'pattern' of the hypophysiotrophic stimulus was important in the control of gonadotrophin secretion. Continuous infusion of LHRH into the hypothalamic lesioned animals resulted in transient increase in gonadotrophin secretion followed by refractoriness of the pituitary gonadotrophs to LHRH. Plasma LH and FSH concentrations then fell to undetectable levels (Belchetz et al., 1978). A similar outcome was obtained when the frequency of LHRH administration was increased from one pulse per hour to two, three or five pulses per hour. Furthermore, decreasing the frequency of LHRH administration not only changed the levels of gonadotrophin in the blood but altered the ratio of LH to FSH (Wildt et al., 1981).

1.5 Relationship of LHRH to LH

The consequence of the rhythmic discharge of LHRH is the pulsatile secretion of LH and FSH by the gonadotrophs into the peripheral circulation. Although Carmel et al. (1976) provided direct evidence that a hypothalamic input was the cause of circhoral LH rhythm, their model in the rhesus monkey necessitated complete pituitary stalk section and it was therefore not possible to correlate LHRH and LH pulses. However, Clarke & Cummins (1982) overcame this by lesioning the hypophysial portal vessels only partially across the pituitary stalk thereby maintaining partial pituitary function. This enabled them to measure LHRH in hypothalamo-hypophyseal portal blood in conscious ovariectomized ewes and LH in the peripheral (jugular) plasma. Their results demonstrated a one-to-one relationship between large LHRH and LH pulses. This was also demonstrated by Levine et al. (1982) using the 'push-pull' perfusion technique in ovariectomized ewes. Both of these techniques also demonstrated LHRH pulses which were not associated with LH pulses and therefore peripheral LH measurements may underestimate hypothalamic activity. The synchrony of LHRH and LH pulses was also demonstrated by Van Vugt et al. (1983) who fitted ovariectomized monkeys with intraventricular cannulae and collected cerebro-spinal fluid from the third ventricle and peripheral blood simultaneously.

1.6 LHRH secretion during the normal human menstrual cycle

The normal pattern of secretion of LHRH, as inferred by hormonal profiles of LH, was examined in detail by Crowley et al. (1985). During the early follicular phase, LH pulses exhibited a low frequency (approximately every 90 min) and moderate amplitude and were almost suspended during sleep. By the mid-follicular phase, the pattern of gonadotrophin release changed markedly. The frequency of LH pulses increased to approximately one every hour (circhoral), the amplitude of each pulse decreased and the sleep-related suspension of pulsatility disappeared. During the late follicular phase, there was a further increase in amplitude of the LH pulses although an increase in frequency could not be defined at this sampling frequency. The pattern of gonadotropin secretion changed again during the early luteal phase with the formation of the corpus luteum. There was a marked slowing of the LH-pulse frequency, with large infrequent pulses of apparently bimodal appearance. These characteristics were exaggerated during the mid-luteal phase. By the end of the luteal phase, the LH pulse frequency continued to slow until only one or two large pulses were observed within a 24h period. The pattern of LH secretion then returned to the more regular, higher amplitude pattern during the transition from the late luteal to the early follicular phase.

1.7 Mechanism of action of LHRH

The action of LHRH is transmitted via binding to specific, high affinity receptors localised on the plasma membrane of pituitary gonadotrophs which then generate intracellular messengers (Clayton, 1987; Conn et al., 1987). This involves the breakdown of polyphosphoinositides to generate inositol triphosphate and diacylglycerol (Clayton, 1987; Conn et al., 1987). These messengers activate protein kinase C resulting in calcium mobilisation and the generation of a cellular response.

The number of LHRH receptors is influenced by the pattern of LHRH exposure, being reduced when exposure is removed and 'up-regulated' by pulses of LHRH (Fraser, 1986; Sandow, 1983; Clayton, 1987). These changes may contribute to changes in gonadotroph responsiveness. As with other peptides, there are large numbers of 'spare' receptors on the pituitary gonadotroph so alteration of post-receptor mechanisms is likely to be of more physiological importance than modulation of receptor numbers (Clayton, 1987).

2. Ovarian control of gonadotrophin secretion.

While LHRH is essential for gonadotrophin secretion by the pituitary gland, the major regulator of LH and FSH secretion is via the negative and positive feedback actions of steroid hormones and possibly inhibin and other related proteins produced by the ovary.

2.1 Oestradiol

2.1.1 Negative feedback

Pituitary gland

The negative feedback action of oestradiol was described by Yamaji et al. (1972), who demonstrated that infusion of a dose of oestradiol, which restored the level of circulating steroid to that found in the early to mid-follicular phase of the menstrual cycle, led to a profound suppression of the circhoral release of LH pulses in ovariectomized rhesus monkeys. This dosage often blocked the next expected secretory episode. Subsequent studies revealed that this inhibition could be sustained for many months (Karsch et al., 1973a; 1973b) and Knobil (1974) concluded that the negative feedback effect of oestradiol played a major role in the regulation of tonic LH secretion during the course of the menstrual cycle in the rhesus monkey. Using their model of the rhesus monkey with hypothalamic lesions to block endogenous LHRH release and restoring hypophysiotrophic support with hourly infusions of LHRH, administration of oestradiol to such animals produced a fall in LH and a decrease in pulsatile LH secretion (Plant et al., 1978). Similar effects of oestradiol at the level of the pituitary gland were noted in intact monkeys in which oestradiol given systemically dampened the increase in serum LH observed after infusion of LHRH (Spies & Norman, 1975). These studies present a strong argument that the negative feedback effects of oestradiol in the monkey are produced by an action on the pituitary gland to suppress the response to LHRH.

Hypothalamus

It was first suggested that the negative feedback effect of oestradiol may act at a site in the hypothalamus by the findings of Bhattacharya et al. (1972). They found that the inhibitory effect of oestradiol on LH pulses in the ovariectomized monkeys could be mimicked by the administration of drugs that blocked alpha-adrenergic or dopaminergic neurotransmission. These drugs have recently been shown to block the rhythmic bursts of hypothalamic multi-unit electrical activity that accompany the rhythmic oscillations of LH, and presumably LHRH, in the ovariectomized monkeys (Kaufman et al., 1985). Further evidence for a hypothalamic site for oestradiol was

provided by the observations of Ferin et al. (1974) in which pulsatile LH release was blocked by microinjections of oestradiol directly into the hypothalamus and from further studies (Ferin et al., 1984) where oestradiol was found to inhibit pulsatile secretion of LHRH. Furthermore, using autoradiography, neurons sequestering oestradiol were concentrated in the same area of brain as LHRH-producing neurons, in the medial basal hypothalamus (Pfaff et al., 1976; Silverman et al., 1982). Alternatively, it has been suggested that oestradiol exerted its inhibitory feedback on both pituitary and central nervous system, with an initial transitory effect on the pituitary followed by a longer lasting effect on the hypothalamus (Weick et al., 1982).

2.1.2 Positive feedback

Pituitary

The initiation of the preovulatory surge of gonadotrophins is the result of positive feedback by oestradiol. When the rising plasma oestradiol concentration during the late follicular phase exceeds a 'threshold' of approximately 200 pg/ml for 48 h, the result is an oestradiol-induced gonadotrophin surge as shown in women (Leyendecker et al., 1979; Monroe et al., 1972; Tsai & Yen, 1971; Yen & Tsai, 1971) and monkeys (Karsch et al., 1973c; Yamaji et al., 1972). Oestradiol has been demonstrated to enhance the response to LHRH at the level of the pituitary gland in the ewe (Clarke & Cummins, 1984; Jackson, 1975) and rhesus monkey (Nakai et al., 1978). It has been suggested that the actions of oestradiol may contribute to the increased amplitude of LH pulses during the surge and its effects are likely to reflect changes in both gonadotrophin biosynthesis and the numbers of LHRH receptors (Karsch, 1987). The number of receptors for LHRH on pituitary cells has been shown to increase at the time of the LH surge in monkeys (Adams et al., 1981), sheep (Crowder & Nett, 1984; Moss et al., 1981) and rats (Savoy-Moore et al., 1980; 1981).

Hypothalamus

A hypothalamic site has also been suggested for the positive feedback actions of oestradiol. An increase in LHRH has been reported in hypophyseal portal blood and in push-pull perfusates from the median eminence during the LH surge in the monkey (Levine et al., 1985; Neill et al., 1977) and sheep (Clarke & Cummins, 1985; Schillo et al., 1985). In the sheep, this effect was described as resulting from an increase in frequency of the LHRH pulse (Clarke & Cummins, 1985) whereas in monkeys it was associated with an increase in pulse amplitude (Levine et al., 1985).

2.2 Progesterone

It is well established that progesterone reduces the frequency of LH pulses in a number of species, including primates (Pohl & Knobil, 1982). This has been demonstrated by the dramatic slowing of LH pulses during the luteal phase of the menstrual cycle in women (Filicori et al., 1984) and monkeys (Norman et al., 1984). This suggests that progesterone mediates its primary neuroendocrine effects by acting upon the frequency of the hypothalamic LHRH pulse generator. In women, progesterone administration in the follicular phase can produce a slowing of LH pulse frequency and an augmentation in LH pulse amplitude (Soules et al., 1984). In the ovariectomized monkey, the frequency of gonadotrophin discharges was reduced by administration of progesterone (Knobil, 1981).

Involvement of opiates

It has been suggested that endogenous opiates may be involved in the actions of ovarian steroids on gonadotrophin. Ferin et al. (1984) concluded that oestradiol and progesterone acted at a hypothalamic site to modulate LHRH signals, oestradiol primarily affecting the amplitude of the pulses while progesterone decreased the frequency. Furthermore, the secretion of β-endorphin into the hypophyseal portal circulation was found to fluctuate cyclically with maximum levels during the luteal phase of the menstrual cycle (Wehrenberg et al., 1982). Following ovariectomy in rhesus monkeys, concentrations of β-endorphin became undetectable (Wehrenberg et al., 1982). Replacement of ovarian steroids restored portal blood concentrations of β-endorphin immunoreactivity indicating that ovarian sex steroids were necessary for the release of hypothalamic β-endorphin (Wardlaw et al., 1982). They also examined the effects of single injections of naloxone, an opiate antagonist, on LH secretion throughout the menstrual cycle and found LH was stimulated only during the luteal phase. This suggested that LH secretion was most suppressed during the luteal phase of the menstrual cycle, when progesterone and β-endorphin levels are maximal.

3. Manipulation of LHRH action

The isolation and structural characterization of LHRH in 1971 by the groups of Schally and Guillemin led to the development of LHRH agonists and antagonists for manipulation of LHRH input to the gonadotroph. These compounds provided an insight into the physiological role of LHRH which resulted in potential clinical and contraceptive applications (for review see Fraser & Baird, 1987; Fraser, 1988a, 1988b).

3.1 LHRH analogues

3.1.1 LHRH agonists

Chemistry and Mechanism of action

LHRH agonists were designed by identifying the sites of enzymatic degradation of LHRH and modifying these positions to increase resistance to peptidases and to enhance affinity of receptor binding. It was found that substitution of a bulky hydrophobic D-amino acid in position 6 resulted in the development of highly active compounds (Table 1). In several compounds the C-terminal glycinamide residue was replaced by an ethylamide group and this was found to have an additive effect (Conn et al., 1987; Vickery & Nestor, 1987). The agonists are 50-200 times more potent than LHRH in releasing gonadotrophins. However, their main application has been the suppression of gonadotrophin release by chronic exposure.

Down-regulation by LHRH agonists

Chronic exposure of the pituitary gonadotrophs to LHRH agonists leads to suppression of pituitary-gonadal function by several complex mechanisms (Fraser, 1988a). The most important of these are :

(i) The over-riding of pulsatile gonadotrophin release.

(ii) Desensitization of the gonadotroph, particularly at the post-receptor level.

(iii) Inducing production of altered forms of gonadotrophin with reduced biological activity.

The initial exposure to LHRH agonist is characterized by marked elevations in serum gonadotrophin concentrations which last for several hours. Continued administration results in decreased pituitary responsiveness which leads to disruption and suppression of the pituitary-ovarian axis. This effect is dependent on the potency of the agonist and the dose, time and frequency of administration. LHRH agonists are inactivated orally and may be administered by injection (for review see Fraser, 1988b), nasal spray (Hardt & Schmidt-Gollwitzer, 1984), pump infusion (Akhtar et al., 1983; Healy et al., 1986) and slow release depots (Walker et al., 1984; 1986). LHRH agonists have proved to have many clinical and therapeutic applications in conditions which respond to removal of the gonadotrophic stimulus to the gonad eg. endometriosis, fibromyomata, menorrhagia, dysmenorrhoea, polycystic ovary syndrome, hirsutism, induction of ovulation with exogenous gonadotrophins, breast

Name	Structure
LHRH	pGlu^{1}, His^{2}, Trp^{3}, Ser^{4},Tyr ^{5}, Gly^{6}, Leu^{7}, Arg^{8}, Pro^{9}, Gly^{10}NH_2
Buserelin (Hoechst)	[D-Ser(But)^{6}, Pro^{9}, Net] 1-9 LHRH
Detirelix (Syntex)	[N-Ac-D-Nal(2)^{1}, D-pCl-Phe^{2}, D-Trp^{3}, D-hArg(Et_2)^{6}, D-Ala^{10}] LHRH

Table 1. Structure of LHRH and the analogues used in the experiments in this thesis- the agonist Buserelin and the antagonist Detirelix.

cancer and in addition may have potential for contraceptive use (for reviews see Fraser & Baird, 1987; Fraser, 1988b).

3.1.2 LHRH antagonists

Chemistry

The development of LHRH antagonists involved a stepwise introduction of hydrophobic residues which block proteolysis, increase LHRH receptor affinity and prolong pharmacokinetics of the molecule in the absence of LH-releasing activity (Vickery & Nestor, 1987; Folkers et al., 1987). The progress in the development of LHRH antagonists was hampered by side effects as a result of histamine release, a phenomenon associated with peptides having positively charged D-amino acids, in particular with antagonists having a D-Arg in position 6 (Vickery & Nestor, 1987). Over 2000 increasingly potent antagonists have been synthesized and current antagonists contain complex unnatural and /or D-amino acids so that only positions 4 and 9 of native LHRH are maintained without change (table 1). The most recently developed LHRH antagonists are long-acting and have produced gonadotrophin-suppressing action lasting for several days after a single administration in monkeys (Leal et al., 1989). This long action may be related to the solubility properties of the antagonists, which are dissolved in propylene glycol and come out of solution after injection, forming a depot. These compounds have low histamine-releasing activity and should be useful in clinical studies.

Mechanism of action

LHRH antagonists exert their action by binding to LHRH receptors with high affinity. The receptors are not internalised or down-regulated and even after treatment with potent antagonists, administration of high doses of LHRH can still induce LH release (Weinberger et al., 1984; Marshall et al., 1986; Chillik et al., 1987; Kenigsberg & Hodgen, 1986). It is possible that it is difficult to occupy all LHRH receptors and activation of the unoccupied receptors may lead to a biological response or a high local concentration of LHRH is capable of displacing LHRH antagonist from the receptors (Fraser, 1988a).

LHRH antagonists have the advantage of inducing immediate inhibition of LH and FSH release and a reduction in gonadal activity without the initial stimulatory phase observed with the LHRH agonists. Administration of antagonists to macaques during the early-or mid-follicular phase for 3-6 days prevented follicular development (Fraser et al., 1987a). Antagonist administration for 3 days around day 7 of the follicular

phase resulted in an abrupt fall in serum oestradiol levels which probably reflected the functional demise of the follicle selected for ovulation (Kenigsberg & Hodgen, 1986; Mais et al., 1986; Fraser et al., 1987a). Ovulation was prevented and a new follicular phase began once recovery from antagonist was complete. Repeated administration of LHRH antagonist as a large weekly dose in rhesus monkeys was associated with rises in serum oestradiol to mid-follicular phase values with absence of ovulation (Kenigsberg & Hodgen, 1986). This treatment in combination with a progestogen (norgestimate) was successful in inhibiting ovulation in the cynomolgus monkey (Danforth et al., 1990). This regimen may prove to be suitable for further development as a contraceptive.

LHRH antagonists as investigative tools during luteal phase.

The development of LHRH antagonists provided a convenient approach for the investigation of the gonadotrophic control of luteal function. Various studies involving the use of LHRH antagonists have established that progesterone secretion by the corpus luteum of women and non-human primates is dependent on pituitary LH secretion. A single injection of LHRH antagonist during the mid- to late- luteal phase in the stumptailed macaque and rhesus monkeys resulted in marked suppression of progesterone secretion (Fraser et al., 1985; Fraser et al., 1986; Vickery, 1986). Furthermore, even during the early-luteal phase, this treatment reduced progesterone secretion (Fraser, 1986). Monkeys treated in the early- to mid-luteal phase with a single injection of LHRH antagonist demonstrated a recovery of normal luteal function after the action of antagonist had subsided. Treatment with LHRH antagonist for 3 days starting during the early-luteal phase caused a permanent suppression of serum progesterone concentrations for the remainder of the cycle (Fraser et al., 1987a). Similarly, in women (Mais et al., 1986) and rhesus monkeys (Collins et al., 1986) LHRH antagonist administration for 3 days or more during the mid- to late- luteal phase resulted in sustained suppression of luteal function. More recently, the inhibitory effects of this treatment during the early luteal phase in women has also been demonstrated (B. Charbonnel, personal communication)

The concurrent administration of human chorionic gonadotrophin (hCG) and LHRH antagonist during the mid-luteal phase resulted in an over-riding of the suppressive effects of the antagonist in women (Mais et al., 1986), stumptailed macaque (Fraser et al., 1987a) and cynomolgus monkey (Collins et al., 1986). The latter study also showing that concurrent administration of FSH with antagonist failed to sustain progesterone concentrations. This suggests that LHRH antagonists could be used to

provide a model in which putative luteotrophic agents could be tested to determine their stimulatory actions.

3.2 Investigation into luteal function by surgical means.

A more invasive approach to investigate luteal function was used in the studies of Hutchison & Zeleznik (1984; 1985). Experiments were conducted using the rhesus monkey as a model, rendered anovulatory by radiofrequency lesions in the arcuate region of the medial basal hypothalamus. Endogenous gonadotrophin secretion and ovulatory cycles were subsequently re-established by chronic pulsatile infusion of LHRH. Endogenous gonadotrophin secretion was interrupted during the early- and mid-luteal phase by stopping the infusion of LHRH (Hutchison & Zeleznik, 1984). Plasma progesterone concentrations declined to undetectable levels resulting in premature menses and it was concluded that the normal functional lifespan of the primate corpus luteum required the presence of circulating pituitary gonadotrophin during the early- and mid-luteal phase. Endogenous gonadotrophin secretion was then interrupted for a 3 day period during the early-, mid- and late-luteal phases (Hutchison & Zeleznik, 1985). During the deprivation period, immunoreactive serum LH was undetectable and was followed by a rapid fall in progesterone. Restoration of gonadotrophin secretion when the gonadotrophin deprivation period was imposed during the early- or mid-luteal phase resulted in resumption of progesterone secretion which continued until the end of the luteal phase. This failed to occur when gonadotrophin secretion was interrupted during the late luteal phase. These results further confirmed that progesterone secretion during the luteal phase was dependent on pituitary gonadotrophin support. The corpus luteum can recover from transient withdrawal resulting in functional luteolysis, but the degree to which luteal function is restored varies with the age of the corpus luteum.

4. Structure and function of the Corpus Luteum

4.1 Formation

A corpus luteum is formed as a result of the action of the preovulatory LH surge on the membrana granulosa and theca interna cells of the mature Graafian follicle. This induces a series of morphological and biochemical changes known as luteinization (Rothchild, 1965; 1981). After ovulation, the basement membrane breaks down and blood vessels from the theca interna invade the cavity of the ruptured follicle. Fibroblasts accompany the vessels and form a dense reticulum network within the granulosa layer as well as an inner fibrous layer which lines the central cavity (McKay

et al, 1961). When mature, the corpus luteum has a diameter of 1.5-2.5 cm and is a yellow structure with festooned contours and a cystic centre filled with a haemorrhagic coagulum (Clement, 1987). On the 8th or 9th day after ovulation, involutional changes begin resulting in luteal regression or luteolysis (Corner, 1956). During this process the granulosa-lutein cells decrease in size, accumulate cytoplasmic lipid and develop pyknotic nuclei (Clement, 1987). There is a decrease in enzymes associated with steroid biosynthesis and an increase in hydrolytic enzymes (Deane et al., 1962) and eventually the cells undergo dissolution and phagocytosis (Adams & Hertig, 1969). This is followed by a progressive fibrosis and shrinkage over several months and conversion into a corpus albicans.

4.2 Cell types within the corpus luteum

Histologically, the human corpus luteum, similar to a variety of other mammalian species, is thought to be composed of at least two morphologically distinguishable cell types, the larger cells (25-50μm) being derived from the granulosa cells of the preovulatory follicle and the smaller cells (15-25μm) being derived from the cells of the theca interna (Corner, 1956). In contrast to the granulosa cells of the follicle, granulosa-lutein cells are larger, polygonal shaped cells with abundant eosinophilic cytoplasm which may contain lipid droplets (Gillim et al., 1969). Granulosa-lutein cells contain a spherical nucleus with one or two nucleoli and the histochemical pattern of these cells varies with the age of the corpus luteum (Deane et al., 1962; Feinberg & Cohen, 1965; Wiley & Esterly, 1976). The theca interna forms an irregular and often interrupted layer around the circumference of the corpus luteum which is several layers in thickness and also surrounds the vascular septa that extend into the centre of the tissue (Clement, 1987). These cells contain a round to oval nucleus with a single prominent nucleolus. In contrast to the granulosa-lutein cells, they have less abundant, more darkly-stained cytoplasm with larger lipid droplets. Both granulosa-lutein and theca-lutein cells contain abundant tubular smooth endoplasmic reticulum, mitochondria with tubular cristae and numerous free ribosomes (Crisp et al., 1970), structural features characteristic of steroid secreting cells. A third cell type, the 'K' cell, is present in small numbers within the theca interna of the mature follicle and within the granulosa-lutein cell layer of the early corpus luteum. These cells are characterized by a stellate shape, deeply eosinophilic cytoplasm and a pyknotic nucleus and persist until menstruation, at which time they degenerate. The cytoplasm is sudanophilic due to the presence of phospholipid (White et al., 1951). 'K' cells do not have the histochemical pattern of steroidogenic cells and have been considered as perivascular macrophages or cells of granulosa or thecal origin which have been

subjected to degenerative changes or altered metabolic activity (Adams & Hertig, 1969; Nelson & Greene, 1958; White et al., 1951).

4.3 Steroid production by luteal cell types

Several different techniques have been employed to attempt to separate and analyse different cell populations within the corpus luteum in different species. Some groups have applied density gradient centrifugation in the cow (Ursely & Leymarie, 1979; Koos & Hansel, 1981), sow (Lemon & Loir, 1977), ewe (Rodgers & O'Shea, 1982) and human (Ohara et al., 1987). Elutriation methods have been employed in the ewe (Fitz et al.,1982) and various groups have used flow cytometry in the cow (Davis et al., 1988a; Alila et al., 1988a, 1988b) and monkey (Hild-Petito et al., 1989). Following separation, studies *in vitro* have shown that both large and small cells from primate corpora lutea synthesize and secrete progesterone and oestradiol. The small cells have a greater capacity for progesterone production in response to LH or hCG than the large cells, the latter containing few if any receptors for LH or hCG (Ohara et al., 1987; Hild-Petito et al., 1989). Oestradiol secretion *in vitro* was stimulated by androgens in large luteal cells but not in small luteal cells from mid luteal phase corpora lutea (Ohara et al., 1987; Hild-Petito et al., 1989): however, androgen production was greater in theca-lutein cells (Macnaughton et al., 1981). Furthermore, small cells responded to LH whereas large cells did not (Hild-Petito et al., 1989; Macnaughton et al., 1981). Sasano et al. (1989) have demonstrated immunocytochemically the presence of P450 aromatase activity in the granulosa-lutein cells and P450 17 α-hydroxylase activity in the theca-lutein cells of the human corpus luteum.

It has been suggested that progesterone production by large luteal cells from the sheep may be regulated by prostaglandins (Fitz et al., 1982; 1984a; 1984b). Moreover, nonhormonal activators of adenylate cyclase increased cyclic adenosine monophosphate (cAMP) accumulation in both large and small luteal cells but did not increase progesterone production by large luteal cells (Hoyer et al., 1984; Hoyer & Niswender, 1986). This suggested that progesterone production by large and small luteal cells in the sheep may be regulated by different mechanisms. Hild-Petito et al. (1989) found that gonadotrophin, prostaglandin E_2 and an analogue of cAMP stimulated progesterone production to the same extent in both large and small luteal cells populations isolated from the monkey corpus luteum. In contrast, Ohara et al. (1987) demonstrated that large and small luteal cells populations from the human corpus luteum exhibited different responsiveness to gonadotrophic stimulation. In the

unstimulated state, large cells were approximately 2-fold more potent in progesterone formation and aromatase activity but only half as potent in androstenedione and testosterone formation as small cells. When stimulated by hCG, small cells responded with significant increases in progesterone, androstenedione and testosterone release but large cells did not. Both cell types secreted oestrone and 17β-oestradiol in the presence of androgen substrate but the addition of FSH significantly stimulated aromatization in large cells. It is possible that the discrepancies between the findings of Hild-Petito et al. (1989) and Ohara et al. (1987) are due to differences in methodology used- the latter study employed Percoll gradient centrifugation whereas flow cytometry was used by Hild-Petito et al. (1989). However, both gonadotrophins and prostaglandins have been shown to stimulate adenylate cyclase activity in the primate corpus luteum (Eyster & Stouffer, 1985; Molskness et al., 1987) suggesting these substances may regulate primate luteal cells via a cAMP-dependent pathway.

5. Control of luteal function

Sheep

In sheep, luteolysis occurs as the result of uterine synthesis and secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)(fig.1.1) (Goding, 1974; Horton & Poyser, 1976; Niswender et al., 1985). A positive feedback loop operates during luteolysis between the ovary and the uterus, $PGF_{2\alpha}$ releases oxytocin from the ovary and oxytocin in turn has the ability to release $PGF_{2\alpha}$ from the uterus (Flint & Sheldrick, 1986) and this cycle continues until luteolysis is complete. The close anatomical proximity of the uterine veins and ovarian arteries permits diffusion of $PGF_{2\alpha}$ directly into the ovarian arteries by a countercurrent diffusion mechanism. This bypasses the general circulation and avoids the rapid destruction of prostaglandin in the lungs (McCracken et al., 1981). Immunization against oxytocin resulted in prolonged luteal cycles in sheep (Sheldrick et al., 1980; Schams et al., 1983) during which oestrus could be induced by injections of exogenous $PGF_{2\alpha}$. When oxytocin injections were administered to sheep, this shortened oestrous cycle length and caused luteal regression (Milne, 1983). It has also been suggested that oxytocin acts to decrease luteal progesterone synthesis (Auletta & Flint, 1988). Active immunization against $PGF_{2\alpha}$ in cyclic ewes results in prevention of luteolysis (Scaramuzzi et al., 1973), providing further evidence for this theory.

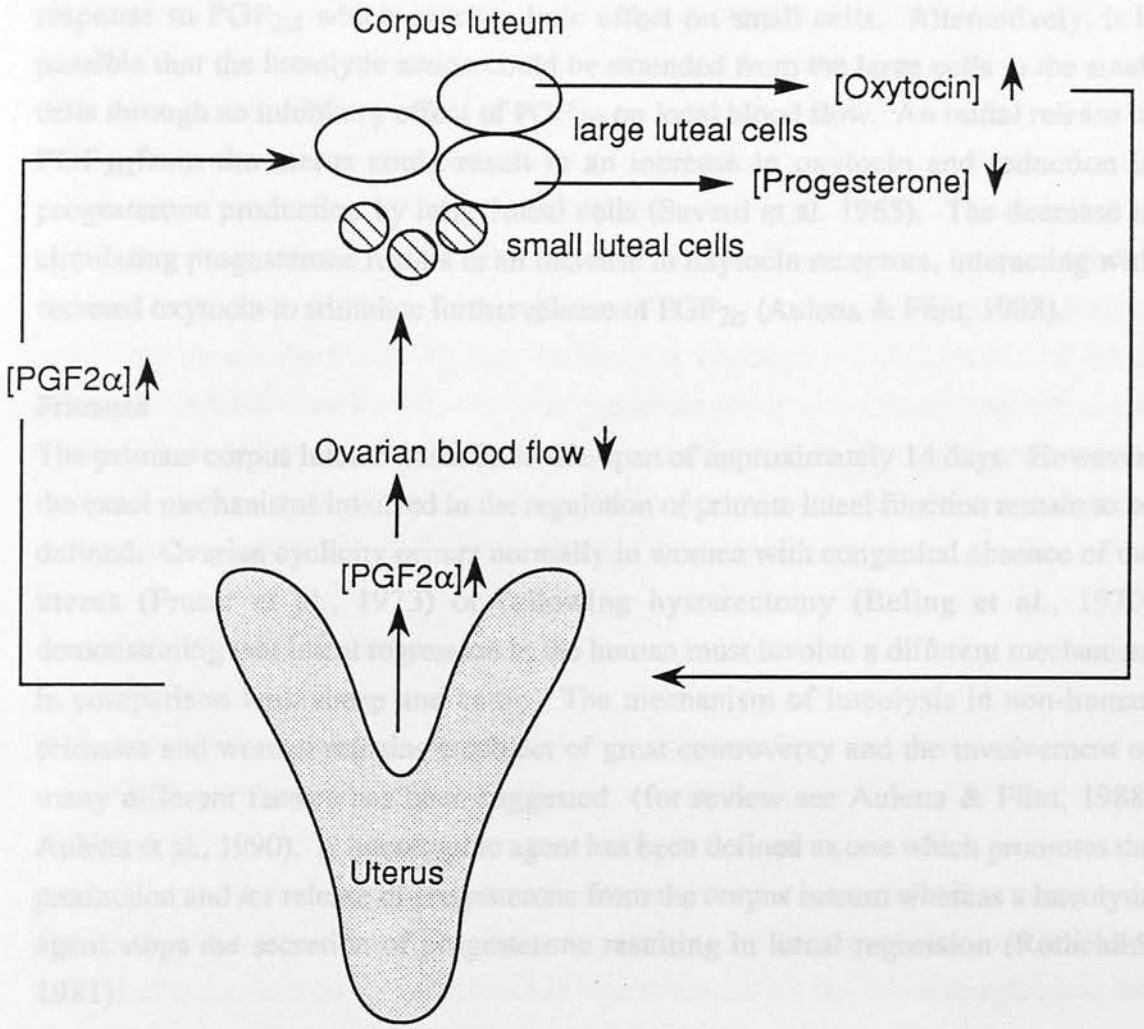


Fig. 1.1 Positive feedback loop of luteolysis in the sheep. Oxytocin released from the large cells of the corpus luteum acts on the uterus to release $PGF_{2\alpha}$. This results in luteolysis either by restriction of ovarian blood flow or by an action on the large cells of the corpus luteum to decrease progesterone and increase oxytocin secretion.

The receptors for $PGF_{2\alpha}$ and oestradiol are primarily associated with the large cells of the corpus luteum in the sheep (Fitz et al., 1982; Schwall & Niswender, 1985; Niswender et al., 1985). It is possible that oxytocin is released from the large cells in response to $PGF_{2\alpha}$ which exerts a lytic effect on small cells. Alternatively, it is possible that the luteolytic action could be extended from the large cells to the small cells through an inhibitory effect of $PGF_{2\alpha}$ on local blood flow. An initial release of $PGF_{2\alpha}$from the uterus could result in an increase in oxytocin and reduction in progesterone production by large luteal cells (Savard et al. 1965). The decrease in circulating progesterone results in an increase in oxytocin receptors, interacting with secreted oxytocin to stimulate further release of $PGF_{2\alpha}$ (Auletta & Flint, 1988).

Primates

The primate corpus luteum has a fixed life-span of approximately 14 days. However, the exact mechanisms involved in the regulation of primate luteal function remain to be defined. Ovarian cyclicity occurs normally in women with congenital absence of the uterus (Fraser et al., 1973) or following hysterectomy (Beling et al., 1970) demonstrating that luteal regression in the human must involve a different mechanism in comparison with sheep and cattle. The mechanism of luteolysis in non-human primates and women remains a subject of great controversy and the involvement of many different factors has been suggested (for review see Auletta & Flint, 1988; Auletta et al., 1990). A luteotrophic agent has been defined as one which promotes the production and /or release of progesterone from the corpus luteum whereas a luteolytic agent stops the secretion of progesterone resulting in luteal regression (Rothchild, 1981).

5.1 Luteotrophic agents

LH/hCG

In most mammalian species studied, LH is necessary for steroid secretion by the corpus luteum. LH and hCG interact with the same cell surface receptor (Dufau & Catt, 1978) which is a glycolipoprotein (Gospodarowicz, 1973). The result of the interaction of a trophic hormone with its receptors includes the formation of intracellular cyclic adenosine monophosphate (cAMP) (for review see Richardson, 1986). cAMP or the protein kinase it activates has a number of steroidogenic actions in steroidogenic cells, one of which is the stimulation of cholesterol side-chain cleavage enzyme complex in the mitochondrion (Richards et al., 1987) which results in increased production of pregnenolone (Hall, 1985). There is evidence to suggest that cAMP is not the sole second messenger system in the corpus luteum. Incubation

of bovine luteal cells in the presence of hCG (Davis et al., 1986a) or LH (Davis et al., 1987a) resulted in elevated inositol-1,4,5-triphosphate (IP_3) and other inositol phosphates and induced rapid, concentration-dependent increases in intracellular Ca^{2+}. These phospholipid-related effects were independent of increased cAMP. There is conflicting evidence concerning stimulation of progesterone *in vitro* by either diacylglycerol (DAG) or IP_3. Phorbol esters, which mimic the actions of DAG, induced a dose- and time-dependent increase in the accumulation of progesterone by rat granulosa cells (Kawai & Clark, 1985) and bovine luteal cells (Hansel & Dowd, 1986; Brunswig et al., 1986; Alila et al., 1988a). These results suggested that phosphatidylinositol-derived second messengers were involved in LH-induced progesterone synthesis. However, Veldhuis & Demers, (1986) found that while phorbol esters activated kinase in porcine granulosa cell *in vitro* , the overall effect was inhibition of cholesterol side chain cleavage and reduction in the rate of progesterone synthesis. The luteotrophic nature of LH was demonstrated *in vivo* by the experiments of Hutchison & Zeleznik (1984; 1985)(see 3.2).

5.2 Luteolytic agents

5.2.1 Role of LH

The idea of involvement of LH in luteolysis originated from the observation that the pulsatile frequency and amplitude of LH in women (Filicori et al., 1984) and rhesus monkeys (Ellinwood et al., 1984) are markedly reduced during the late luteal phase, in comparison with the early and mid-luteal phase. Further evidence for this theory was provided by results obtained from Schoonmaker et al.(1984) and Ellinwood & Resko (1983) where a decrease in bioactive LH was noted during the late luteal phase in the rhesus monkey. However, this observation has not been observed in women (Beitins & Dufau, 1986). Furthermore, Hutchison et al. (1986) reduced the LH pulse frequency to 1 pulse every 8 hours in rhesus monkeys with hypothalamic lesions and luteal function was maintained. The results from these studies concluded that a decrease in pulsatile LH secretion alone was not sufficient to promote luteolysis but may contribute to declining luteal function in primates.

5.2.2 Role of the LH receptor

The possibility that spontaneous luteolysis results from the inability of LH to bind to its specific receptor on the corpus luteum has also been investigated. In the sheep, the number of occupied and unoccupied LH receptors correlates with the weight of the corpus luteum and with luteal and serum levels of progesterone. Furthermore, the total and occupied concentrations of LH receptor are more highly correlated with the

secretion of progesterone than with circulating LH (Niswender et al., 1985). Diekman et al.(1978) demonstrated a decrease in both serum progesterone and concentration of LH receptor as luteolysis approaches. However the fall in progesterone preceeded the fall in LH receptor concentrations and they concluded that receptor loss is therefore not an initial step in luteolysis. Similar findings have been reported in non-human primates and in women (McNeilly et al, 1980; Bramley et al., 1987; Rao et al., 1977a; Halme et al., 1978).

5.2.3 $PGF_{2\alpha}$

There is some evidence that $PGF_{2\alpha}$ is a luteolysin in non-human primates and women. Intraluteal infusions of $PGF_{2\alpha}$ in rhesus monkeys resulted in luteal regression (Auletta et al., 1984a). Transient reductions in circulating progesterone have been reported in rhesus monkeys after intra-luteal (Sotrel et al., 1981) or systemic (Kirton et al., 1970) administration of pharmacological doses of $PGF_{2\alpha}$. Similar results have been reported in women (Korda et al., 1975; Wentz & Jones, 1973) and also following intra-arterial infusion (Auletta et al., 1973) and intraluteal injection of $PGF_{2\alpha}$ (Sotrel et al., 1981). However, failure of systemically administered $PGF_{2\alpha}$ in causing luteolysis has also been reported which may be accounted for by refractoriness of the corpus luteum at certain times (Stouffer et al., 1979) or by rapid metabolism of prostaglandin during the first passage through the pulmonary circulation.

$PGF_{2\alpha}$ is synthesized by the primate (Wilks et al. 1972; Johnson et al., 1988) and human corpus luteum (Challis et al., 1976) and $PGF_{2\alpha}$ receptors have been identified in human luteal membrane preparations (Powell et al., 1974; Rao et al., 1977b). The concentration of $PGF_{2\alpha}$ in human luteal tissue during luteolysis has been shown to be higher than in any other stage of the cycle by some groups (Shutt et al., 1976; Patwardhan & Lanthier, 1980; Vijayakumar & Walters, 1983). However, this has been contradicted by others (Challis et al., 1976; Swanston et al., 1977). It has been shown that $PGF_{2\alpha}$ can stimulate progesterone secretion from large and small luteal cells (Hild-Petito et al., 1989). Administration of ibuprofen, a cyclo-oxygenase inhibitor, directly into the rhesus monkey corpus luteum during the mid-luteal phase, resulted in higher progesterone levels and a slightly prolonged luteal phase (Auletta et al., 1988a). Furthermore, Sargent et al.(1988) observed premature luteolysis using intraluteal infusion of meclofenamate, a non-steroidal anti-inflammatory drug which irreversibly blocks the enzyme cyclooxygenase, during the mid-luteal phase, suggesting a luteotrophic role for prostaglandins at this time. The major circulating metabolite of $PGF_{2\alpha}$ is 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ($DHKF_{2\alpha}$) and levels of this

metabolite have been shown to be significantly higher in ovarian venous effluent ipsilateral to the corpus luteum, when compared to the contralateral side during the late luteal phase in the rhesus monkey (Auletta et al., 1984a). Concurrent measurements of $DHKF_{2\alpha}$ in peripheral plasma failed to show any change in concentrations at the time of luteolysis (Auletta et al., 1984a). This has also been demonstrated in women (Van Orden et al., 1977).

Taken together, this evidence suggests that $PGF_{2\alpha}$ may be involved in luteolysis through appropriately timed luteal synthesis (Auletta et al., 1990). It is possible that $PGF_{2\alpha}$ may act via a direct action on the luteal cells and/or decreased or redistribution of blood flow away from the corpus luteum. However, the mechanisms of such a process remains to be determined.

5.2.4 Role of Oestradiol

It has been suggested that oestradiol is also a physiological luteolysin in primates. Oestrogens are synthesized by the monkey and human corpus luteum throughout the luteal phase (Savard et al., 1965; Butler et al., 1975; Richardson & Masson, 1981). Oestradiol suppresses basal and/or gonadotrophin-stimulated progesterone production by monkey (Stouffer et al., 1977) and human (Williams et al., 1979) luteal cells *in vitro*. Exogenously administered oestrogens given systemically or locally into the corpus luteum cause premature, functional luteolysis (Auletta et al., 1972; Karsch et al., 1973d; Karsch & Sutton, 1976; Gore et al., 1973). Furthermore, Ravindranath & Moudgal (1987) reported that the anti-oestrogen, tamoxifen, prolonged luteal phase length in the bonnet monkey and suggested this effect may be mediated by an increase in basal concentrations of LH or by antagonizing a natural luteolytic action of oestradiol. However, no statistical analysis of progesterone or oestradiol levels was provided and no additional hormonal data were assessed. In a later study by Olive et al. (1990) it was reported that low or high doses of tamoxifen had no effect on luteal phase length in the cynomolgus monkey. No differences in gonadotrophin levels were observed and oestradiol and progesterone levels remained unaltered. It was concluded from this study that tamoxifen had no effect on spontaneous luteal function in the cynomolgus monkey.

Three sites of action have been proposed for oestrogen-induced luteal regression: hypothalamus, pituitary and corpus luteum.

Hypothalamus

Maruncic & Casper (1987) demonstrated an increase in LH pulse frequency but not in amplitude after administration of an anti-oestrogen, clomiphene citrate, to normal women in the luteal phase, suggesting a central action probably involving hypothalamic LHRH release. Following treatment, elevations in serum oestradiol and progesterone levels was observed with a lengthening of the luteal phase.

Pituitary

In the rhesus monkey, intraluteal administeration of oestradiol resulted in depressed progesterone secretion without any detectable changes in circulating LH (Stouffer et al., 1979; Karsch & Sutton, 1976). However, some studies have noted a decrease in peripheral LH following administration of oestradiol (Schoonmaker et al., 1984; Ellinwood & Resko, 1983; Karsch et al., 1973d) and the discrepancies in these findings are probably dose-related (Auletta et al, 1985).

Corpus Luteum

Oestradiol did not inhibit cyclic adenosine monophosphate (cAMP) accumulation by hCG in cultures of human luteal tissue (Williams et al., 1979; Hahlin et al., 1986) or the action of dibutyryl-cAMP on progesterone synthesis (Williams et al., 1979). These studies suggest that oestradiol may act directly at a site after adenylate cyclase, possibly on the enzymes involved in steroidogenesis (Depp et al., 1973; Caffrey et al., 1979). However, Hild-Petito et al. (1988) failed to detect oestrogen receptors in monkey corpora lutea by immunocytochemistry which does not support the concept of a local receptor-mediated role for oestrogen in inducing luteal regression in primates. The authors suggested that the control of steroid receptor expression is not via an oestrogen receptor-mediated pathway and may be directly or indirectly regulated by gonadotrophin.

Another theory for oestrogen induced luteolysis is through stimulating local production of $PGF_{2\alpha}$ through the corpus luteum or ovary. This is suggested from studies where infusion of oestradiol *in vivo* resulted in release of $PGF_{2\alpha}$ from the ovary bearing the corpus luteum in rhesus monkeys (Auletta et al., 1978) whereas acute in vitro incubation of luteal cells with oestradiol failed to cause release of prostaglandin (Johnson et al., 1988). Studies using incubations of dispersed primate luteal cells (Stouffer et al., 1977; Laherty et al., 1985; Williams et al., 1979) and luteal tissue (Hahlin et al., 1986) have shown that exposure to oestradiol depresses both basal and

LH/hCG stimulated progesterone production, indicating a direct action on the corpus luteum.

5.2.5 Oxytocin

There is a great deal of evidence demonstrating the presence of oxytocin in the ruminant corpus luteum (Flint & Sheldrick, 1982; Wathes & Swann, 1982) and its role in luteolysis in sheep and cattle (Flint & Sheldrick, 1986). However, the production of oxytocin in primate luteal tissue is still very controversial. Many groups have attempted to measure oxytocin in the primate corpus luteum by various methods;

(i) High pressure liquid chromatography (HPLC)/radioimmunoassay (RIA). Oxytocin was first reported in human corpora lutea by Wathes et al. (1982) at much lower levels in comparison with the concentration found in the sheep by HPLC and RIA. These results were later confirmed by other groups using HPLC and different radioimmunoassays (Khan-Dawood & Dawood, 1983; Dawood & Khan-Dawood, 1986; Schaeffer et al., 1984). However, Richardson (1986) was unable to detect oxytocin in the human corpus luteum by HPLC/RIA. In addition, Auletta et al (1988b) have been unable to detect oxytocin in ovarian venous blood in the rhesus monkey and in the corpus luteum of the marmoset whereas Khan-Dawood et al. (1989) have detected oxytocin in human peripheral plasma and ovarian blood, the concentration of oxytocin being highest in the vein draining the ovary bearing the corpus luteum.

(ii) mRNA analysis. A single observation has been reported of the presence of oxytocin-neurophysin prohormone mRNA in human luteal tissue by dot-blot analysis (Rehbein et al., 1986). However, Auletta et al.(1988b) did not detect oxytocin in luteal tissue by Northern blotting using a cDNA probe derived from the same sequence as Rehbein et al.(1986) where sheep tissue gave positive results.

(iii) Oxytocin has been detected immunocytochemically in the human (Khan-Dawood, 1987b), cynomolgus monkey (Khan-Dawood et al., 1983) and baboon corpus luteum (Khan-Dawood, 1986) and again these results have been contradicted by Auletta et al (1988b).

It has also been suggested that oxytocin could act in a paracrine manner to control luteal function. Oxytocin biosynthesis has been demonstrated in serum-free cultures of human granulosa cells (Plevarkis et al., 1990) and oxytocin has also been shown to inhibit progesterone secretion by human luteal cells *in vitro* (Bennegard et al., 1987). Furthermore, oxytocin infused directly into the corpus luteum of the rhesus monkey resulted in luteolysis (Auletta et al., 1984b) suggesting that oxytocin may be luteolytic via local production and action within the corpus luteum.

The discrepancies in the findings of oxytocin in the primate corpus luteum could be explained firstly by the fact that if the primate corpus luteum contains oxytocin, it is present in extremely low concentrations. This would explain why there is no rise in circulating concentrations of oxytocin during the luteal phase of the human menstrual cycle (Amico et al., 1981). Secondly, oxytocin may not be present throughout the life of the corpus luteum. Although Dawood & Khan-Dawood (1986) did report oxytocin to be present throughout the luteal phase, they reported it to be present only between days 19 and 24 of the cycle by immunocytochemistry (Khan-Dawood, 1987b). Finally, the discrepancies in these results could be explained by the differences in methodology between groups, especially the specificity of the antibodies used in radioimmunoassays. This was noted by Fuchs (1988) who demonstrated differences in specificity between different antibodies. In this study, Fuchs found the antibody used by Dawood et al. (1978) detected a mid-cycle peak in oxytocin concentrations from 8 normal women whereas the antibody produced by Morris et al.(1980), did not reveal any rise in oxytocin during this period. Furthermore, the antibody from Dawood et al. (1978) was found to cross-react with an oestrogen-induced oxytocin metabolite. This peptide was first noted by Amico et al.(1985) who found it could induce large molecular weight proteins into the circulation, induce enzymes to cleave oxytocin and induce the synthesis of another unidentified secretory product. In contrast, Flint et al.(1988) found purified acid extracts from human corpus luteum to contain a peptide of close resemblance to haemoglobin which may question the specificity of the radioimmunoassays. In a recent study (Ivell et al., 1990), mRNA copies of the oxytocin gene were detected in both human and baboon corpus luteum. However, the levels of mRNA present were found to be extremely low and the authors suggested that oxytocin may therefore play a modulatory role in ovarian steroidogenesis, perhaps in conjunction with other local factors.

6.0 Inhibin and related peptides

While it is accepted that ovarian cyclicity is dependent on the negative feedback actions of progesterone and oestradiol at the level of the hypothalamus and pituitary, for many years evidence has been provided for a non-steroidal factor named inhibin, which specifically suppresses the secretion of FSH by a direct action on the anterior pituitary. Indeed, the concept of inhibin was proposed even before LH and FSH were defined as separate entities. Inhibin was first described by McCullagh (1932) as a water soluble extract of bovine testis which had the capacity to suppress the formation of castration cells in the anterior pituitary gland. In 1976, de Jong and Sharpe provided the first direct evidence for the presence of inhibin when they discovered that steroid-free

extracts of ovarian follicular fluid prevented the post-castration rise in FSH but not LH.

6.1 Isolation and purification

The subsequent isolation of inhibin proved difficult due to the hydrophobic nature of the molecule and the low levels of inhibin present in tissues. Hence there was a delay in development of suitable assays with adequate sensitivity for measurement of inhibin in the circulation. The initial isolation of inhibin was achieved from bovine follicular fluid as a 58 kDa glycoprotein consisting of two disulphide-linked subunits of 43 kDa and 15 kDa (Robertson et al., 1985). The introduction of a pH precipitation step during the purification procedures led to the isolation of a 31 kDa form consisting of subunits of 20 kDa and 11 kDa (Robertson et al., 1986). A similar form also from bovine follicular fluid was reported by Fukada et al. (1986). Miyamoto et al. (1985) reported the isolation of a 32 kDa form from porcine follicular fluid consisting of subunits of 20 kDa and 13 kDa. This finding was confirmed by Ling et al. (1985) and Rivier et al. (1985). Two forms of inhibin (α and β) were isolated from bovine (Robertson et al., 1985) and porcine follicular fluid (Ling et al., 1985). The amino acid sequence of inhibin was elucidated from the cloning of genes controlling the production of the mRNA for inhibin subunits from bovine and porcine sources. Two different forms of β-subunit were detected (βA and βB) and each of the subunits was found to be encoded by separate genes (Mason et al., 1985; Forage et al., 1986). The structures of the genes encoding for the inhibin subunits in human, ovine and rat inhibin are now known (Mason et al., 1986; Mayo et al., 1986; Stewart et al., 1986; Bardin et al., 1987; Woodruff et al., 1987).

6.2 Structure of inhibin and inhibin-like peptides

Inhibin is a disulphide-linked heterodimer consisting of two dissimilar subunits termed α and β, which are joined by disulphide bonds (Robertson et al., 1985). Inhibin dimers exists as inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$) (fig1.2). Both forms consist of identical α-subunits and different β-subunits (Ling et al., 1985). The β_A subunit is one amino acid longer than the β_B subunit and there is 70% homology between the β subunits (Mason et al., 1985). The inhibin subunits belong to the transforming growth factor β (TGF-β) family of proteins which include Müllerian inhibiting substance, decapentaplegic gene complex of Drosophila and the VG1 gene in Xenopus (for review see de Kretser & Robertson, 1989). There is approximately 70% structural homology between the α and β chains of inhibin within a species. Between

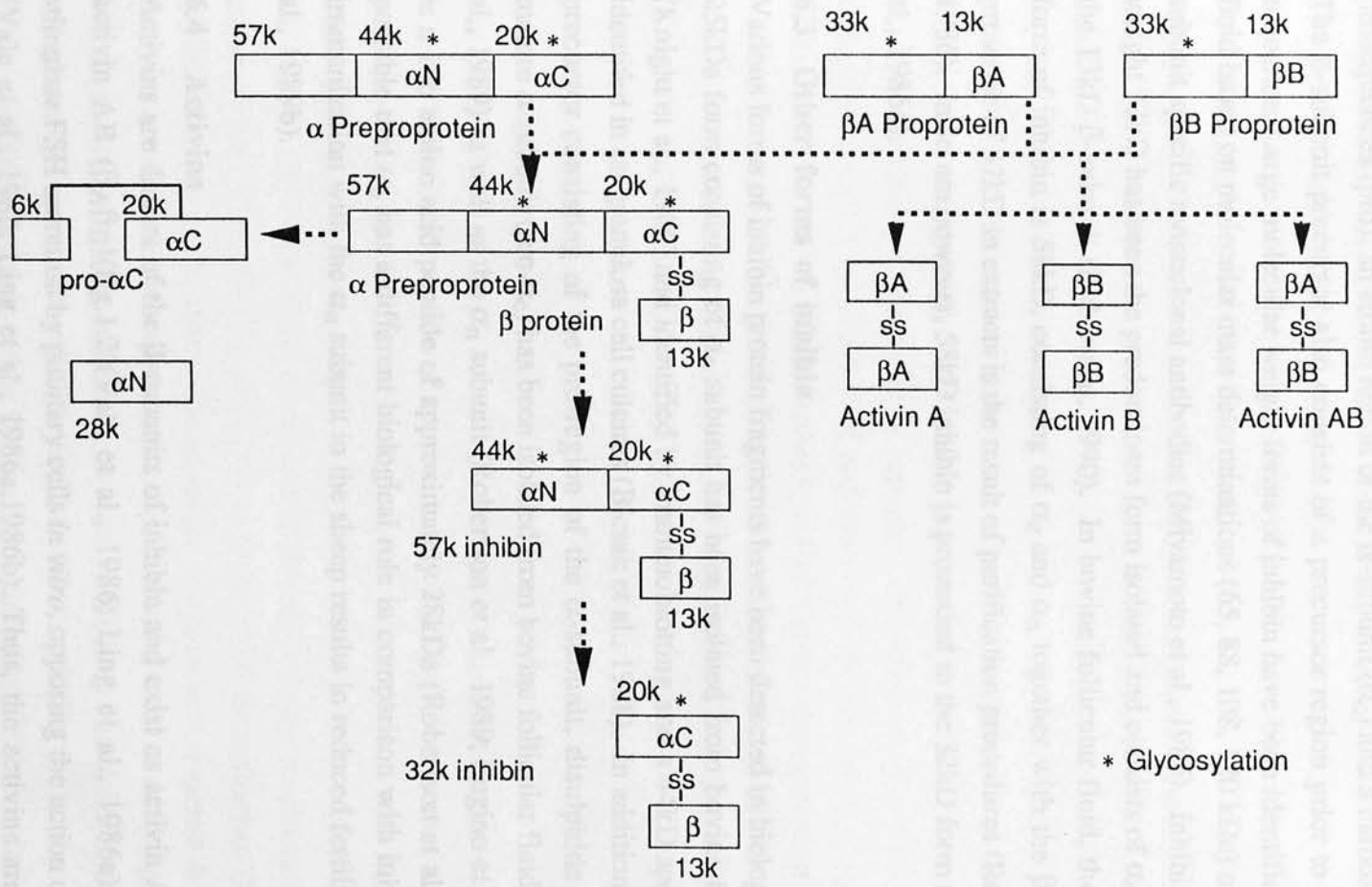


Fig. 1.2 Biosynthesis of inhibin and activin

species, there is approximately 82% homology between the human, bovine and porcine α-subunits whereas the β-subunit DNA sequence is completely conserved (Stewart et al., 1986).

The inhibin α-subunit is initially synthesized as a preproprotein and the β-subunit as a proprotein . The α-subunit precursor consists of three segments; a signal peptide and prosequences (pro), an amino region of the α-subunit (α_c) and a carboxy region (α_n). The β-subunit precursor also consists of a precursor region prior to the β-subunit sequence. Large molecular weight forms of inhibin have been identified in follicular fluid based on molecular mass determinations (65, 88, 108, 120 kDa) using α- and β-subunit specific monoclonal antibodies (Miyamoto et al., 1986). Inhibin of molecular weight 32kD has been the predominant form isolated and consists of α_c together with the 13kD β-subunit (Robertson, 1990). In bovine follicular fluid, the predominant form of inhibin is 58kD, consisting of α_c and α_n together with the β-subunit. The presence of 32kD in extracts is the result of purification procedures (Robertson et al., 1986). In serum however, 58kD inhibin is processed to the 32kD form (McLachlan et al., 1986a).

6.3 Other forms of inhibin

Various forms of inhibin protein fragments have been detected in biological fluids. A 25kDa form consisting of α_c subunit has been isolated from bovine follicular fluid (Knight et al., 1989) and identified by immunoblotting and a 44kD species has been identifed in rat granulosa cell cultures (Bicsak et al., 1988). In addition, an α-subunit precursor consisting of the pro-region of the α-subunit, disulphide linked to the mature α-subunit (pro-α_c) has been isolated from bovine follicular fluid (Robertson et al., 1989) as well as the α_n subunit (Robertson et al., 1989; Sugino et al., 1989). α_n is a 166 amino acid peptide of approximately 28kDa (Robertson et al., 1989). It is possible that α_n has a different biological role in comparison with inhibin as active immunization with the α_n subunit in the sheep results in reduced fertility (Findlay et al., 1989b).

6.4 Activins

Activins are dimers of the β-subunits of inhibin and exist as activin A ($\beta_A\beta_A$) and activin AB ($\beta_A\beta_B$)(fig.1.2)(Vale et al., 1986; Ling et al., 1986a). Both forms stimulate FSH secretion by pituitary cells *in vitro*, opposing the action of the inhibins (Vale et al., 1986; Ling et al., 1986a,1986b). Thus, the activins are endogenous inhibin antagonists. More recently activin B ($\beta_B\beta_B$) has been synthesized in

recombinant form (Mason et al., 1989). Activin A has been shown to promote differentiation of a variety of erythroleukemic cells *in vitro* and synergize with erthropoietin in stimulating erythroid colony formation in primary bone marrow cultures (Eto et al., 1987; Kitaoka et al., 1987; Yu et al., 1987; Broxmeyer et al., 1988). Furthermore, a second TGFβ subunit has been identified which also appears in both homodimeric and heterodimeric configurations (Chiefetz et al., 1987) and it is possible that activin and TGFβ act synergistically to antagonize the actions of inhibin in a paracrine manner (Sugino et al., 1988).

6.5 Follistatin

Another group of proteins which affect FSH release but are structurally distinct from inhibin have been isolated from bovine and porcine follicular fluid. These proteins are termed FSH-suppressing proteins (FSP) or follistatin (Robertson et al., 1987; Ueno et al., 1987). Three molecular weight forms have been identified with molecular weights of 39, 35 and 31kD and all have a common NH_2-terminal sequence. The gene sequences encoding for rat, human and porcine FSP have been isolated and share no structural homology with inhibin subunits. However, they exhibit homology to human pancreatic trypsin inhibitor and epidermal growth factor (Esch et al., 1987; Shimasaki et al., 1988; 1989). mRNA to FSP has been identified in ovary, kidney and brain (Shimasaki et al., 1989) and FSP has been shown to inhibit luteinization of granulosa cells *in vitro* (Xiao et al., 1990). Follistatin has been implicated as a binding protein for activin (Nakamura et al., 1990).

6.6 Measurement of Inhibin

6.6.1 Radioimmunoassay

The measurement of inhibin in biological fluids by RIA is complicated by the presence of these various forms of non-bioactive α-subunits which may cross react with assay antisera. Two types of radioimmunoassay have been developed. The first type of assay is based on antisera raised to the synthetic amino terminal region (amino acids 1-26, 1-30, 1-32) of the 20kD α-subunit of inhibin and the results are expressed in terms of mass of synthetic peptide (Rivier et al., 1986; Schanbacher 1988; Sharpe et al., 1988; Knight et al., 1989). These assays utilise iodinated amino terminal peptide or intact inhibin as tracer. While it is stated that activin, transforming growth factor β and Müllerian inhibitory substance cross-react minimally in these assays, these peptide assays will detect α-subunit as well as the whole inhibin molecule. These assays express sufficient sensitivity for the measurement of inhibin in peripheral plasma of several species excluding human.

The second type of assay is based on antisera raised against native 58kD or 30-32kD whole bovine inhibin molecule prepared from bovine follicular fluid using iodinated whole bovine inhibin as tracer and standards prepared from partially purified human follicular fluid (McLachlan et al., 1986a; Hasegawa et al., 1987; Robertson et al., 1988a, 1988b). Under optimal conditions, the displacement caused by inhibin-free serum is minimal (±10%) although it is usual practice to supplement the standard with an equal volume of serum from gonadectomized animals or postmenopausal women. These assays show little or no cross-reaction with a range of inhibin-related proteins including activin, transforming growth factor-β, FSH-suppressing protein and the α- and β-subunits of inhibin obtained after reductive alkylation. However, cross reaction with other forms of inhibin has been suggested (Robertson et al., 1989; Schneyer et al., 1990). These assays have been used extensively in the measurement of serum inhibin in the human (for review see de Kretser & Robertson, 1989), stumptailed macaque (Fraser et al., 1989; Fingscheidt et al., 1989), sheep (Findlay et al., 1990a), pig, cow (Hasegawa et al., 1987;1988), rat (Hasegawa et al., 1987; Robertson et al., 1988b), goat, cat, dog and horse (Hamada et al., 1989).

A further type of assay system is currently being investigated. A two-site immunoradiometric assay (IRMA) using antibodies raised against the synthetic amino terminal region (amino acids 1-32) of the α-subunit together with antibodies raised against amino acids 97-113 of the β-subunit of inhibin is currently being developed (A.S.McNeilly, unpublished observations). This assay system would presumably detect only the whole inhibin molecule.

6.6.2 Bioassays

The biological assays for inhibin involve the use of monolayer cultures of rat or ovine dispersed pituitary cells and the measurement by radioimmunoassay of the FSH content of the cells (Scott et al., 1980; Scott & Burger, 1981), the release of FSH and LH into the culture medium (Steinberger & Steinberger, 1976, De Paolo et al., 1979, Tsonis et al., 1986) or the FSH and LH secreted in response to LHRH stimulation (de Jong et al., 1979; Eddie et al., 1979). There are many complications associated with the use of bioassays. The rat monolayer cultures are relatively insensitive and are not suitable for the measurement of inhibin in plasma. Sheep pituitary cells are an order of magnitiude more sensitive and have been used in the measurement of inhibin in peripheral and ovarian venous plasma (Tsonis et al., 1986). All samples used in this system must be stripped of steroids to prevent any effects of oestradiol and testosterone on FSH secretion (Eddie et al., 1979). However, the serum of some

species eg. stumptailed macaque has been shown to be toxic in this bioassay system (C.G.Tsonis, R.Leask & H.M.Fraser, unpublished observations). It is possible that the presence of activin and follistatin may influence results obtained in bioassays. In inhibin radioimmunoassays, FSP does not cross react with antisera (Robertson et al., 1987) and the levels of activin and FSP in ovarian and testicular extracts are believed to be lower (10-20%) than those of inhibin, suggesting that bioassay estimates of inhibin activity are not markedly influenced by FSP (Robertson, 1990).

6.7 Physiological role of inhibin in the female.

6.7.1 Follicle

Evidence from early studies suggested that the developing follicle was the major source of circulating inhibin in all species investigated at that time. The demonstration by de Jong and Sharpe (1976) that follicular fluid was a potent source of inhibin activity was followed by the findings of Erickson & Hsueh (1978) who showed that rat granulosa cells in culture produced bioactive inhibin. These results were confirmed in various studies using both *in vitro* bioassay and radioimmunoassay in the rat (Hermans et al., 1982; Croze & Franchimont, 1984; Sander et al., 1984), cow (Henderson & Franchimont, 1981), pig (Channing et al., 1982), monkey (Noguchi et al., 1987) and human (Channing et al., 1984). Furthermore, the amount of inhibin produced by granulosa cells from large follicles is greater than that from small follicles (Channing et al., 1982) and there is a greater concentration of inhibin in the follicular fluid of larger follicles (Tsonis et al., 1983). The latter study also demonstrated a significant correlation of inhibin with oestradiol concentrations and inhibin activity within the follicle. Similarly, a good correlation was obtained between circulating levels of oestradiol and inhibin in women undergoing ovarian hyperstimulation for *in vitro* fertilization (IVF) (McLachlan et al., 1986c). The latter study also showed that the concentrations of oestradiol and inhibin were closely correlated with the number of large antral follicles. The stimulation of inhibin by FSH is also observed during the normal human menstrual cycle where a small rise in inhibin is noted during the peri-ovulatory period (Tsonis et al., 1988; McLachlan et al., 1990). These results suggested that inhibin secretion by the follicle is dependant on continued stimulation by FSH. The increasing amounts of oestradiol secreted by the pre-ovulatory follicle decreases the plasma concentrations of FSH by negative feedback. As a consequence, inhibin secretion declines until the sharp increase in FSH at the LH surge, when inhibin also rises (McNeilly et al., 1988).

FSH has been shown to be the predominant endocrine regulator of the production of inhibin by granulosa cells, while LH at low doses is able to cause release of inhibin from granulosa cells after their exposure to FSH (Bicsak et al., 1986; Zhiwen et al., 1988a). Higher doses of LH/hCG inhibit PMSG/FSH-induced production of inhibin *in vitro* (Zhiwen et al., 1987a; 1988a) and *in vivo* (Lee, 1983). Furthermore, analysis of ovarian mRNA has shown a relative increase in the amount encoding for the α (Davis et al., 1986b) and β_A subunits of inhibin (Davis et al., 1988b) following treatment of rats *in vivo* with PMSG.

6.7.2 Corpus luteum

The development of radioimmunoassays with sufficient sensitivity for the detection of circulating inhibin concentrations in human plasma led to the first indication that the corpus luteum was a major source of immunoreactive inhibin. Using a heterologous radioimmunoassay developed by Monash University, Melbourne, Australia (McLachlan et al., 1986a; McLachlan et al., 1987a) it was demonstrated that serum concentrations of immunoreactive inhibin were highest during the luteal phase in the normal human menstrual cycle (McLachlan et al., 1987a; Buckler et al., 1988). Similar findings were obtained in the Old World primate, the stumptailed macaque (Fraser et al., 1989). This view was also supported by *in vitro* studies demonstrating that human luteinized granulosa cells in culture have the capacity to produce inhibin (Tsonis et al., 1987a, 1987b). Furthermore, the mRNA for the α-subunit has been identified in RNA isolated from luteal tissue from human (Davis et al., 1987b) and primates (Hillier et al., 1989; Basseti et al., 1990). In addition, evidence for both α and β_A subunit expression using *in situ* hybridization has been demonstrated in the primate corpus luteum (Schwall et al., 1990).

In contrast with the human, current evidence from non-primate species indicates that the corpus luteum is not the source of circulating inhibin. Studies in the cow (Hasegawa et al., 1987), sheep (McNeilly et al, 1989) pig (Hasegawa et al., 1988) and rat (Rivier et al., 1989; Taya et al., 1989) fail to show a rise in inhibin concentrations during the luteal phase. Inhibin has been detected immunocytochemically in the recently formed corpus luteum of the rat (Cuevas et al., 1987) and low levels of α-inhibin mRNA expression are present at this stage of development. However both immunocytochemical staining and mRNA expression were absent in the mature corpus luteum (Davis et al., 1986b; Woodruff et al., 1987;

Meunier et al, 1988a). In corpora lutea of cattle and sheep, α-inhibin mRNA was found to be undetectable (Rodgers et al., 1989; Torney et al., 1989).

6.7.3 Paracrine role

Two paracrine actions of inhibin have been reported. Firstly, inhibin has been shown to enhance LH-induced androgen production by rat thecal cell preparations, an effect which is attenuated by activin (Hseuh et al., 1987). Secondly, inhibin (bovine $\alpha\beta_A$) has been shown to inhibit meiotic maturation of oocytes obtained from immature rats (O et al., 1989). As well as being under the endocrine control of FSH, inhibin may also be controlled via autocrine and paracrine mechanisms. IGF-1 either alone or in synergism with FSH, stimulated inhibin production by rat granulosa cells in a time- and dose-dependent manner (Bicsak et al., 1986; Zhiwen et al., 1987a). There is now substantial evidence for hormonally-regulated (FSH, growth hormone and oestradiol) production of IGF-1 by granulosa cells suggesting that the actions of IGF-1 may control inhibin via an autocrine mechanism (Findlay et al., 1990b). Furthermore, TGF-β caused a dose-dependent increase in basal and FSH-stimulated production of inhibin (Zhiwen et al., 1988b). The effects of TGF-β and FSH were additive, TGF-β increasing the sensitivity of granulosa cells to FSH. As rat and bovine tissue produce TGF-β this suggests a paracrine action on inhibin production by granulosa cells.

6.7.4 Extra-gonadal actions of inhibin and activin

Meunier et al. (1988b) demonstrated α- and β-subunits in a number of extra-gonadal locations such as brain, spleen, adrenal gland, pituitary, kidney and bone marrow, supporting the view that the inhibins and activins may have more widespread actions in addition to those in the reproductive system. Eto et al. (1987) implicated activin A as an erythroid differentiation factor in leukemic cell lines. Furthermore, Yu et al. (1987) noted that activin synergized with erythropoietin in stimulating erythroid differentiation, an action opposed by inhibin. Thus, activin could be produced in normal bone marrow cells and could be regulated by inhibin from other sources. An immunomodulatory role for inhibin and activin has also been suggested. Inhibin stimulates and activin A inhibits the uptake of ^{3}H-thymidine into rat thymocytes in the presence and absence of lectins (Hedger et al., 1989). In contrast, bovine activin A stimulates ^{3}H-thymidine incorporation into 3T3 fibroblasts demonstrating that the effects on cell proliferation are cell specific (Hedger et al., 1989).

The demonstration of mRNA for inhibin in the brain and spinal cord (Meunier et al., 1988b) was confirmed by Roberts et al. (1989) who found that α- and β-subunits

were co-localized in the majority of FSH- and LH-immunoreactive gonadotrophs in rat brain. Ovariectomy dramatically increased the size and number of immunopositive gonadotrophs and the mRNAs encoding the subunits also increased. These increases were prevented by oestrogen replacement suggesting that expression of these subunits is regulated by ovarian hormones. Furthermore, Sawchenko et al.(1988) demonstrated immunohistochemical staining for inhibin β-subunit in neurons of the nucleus tractus solitarius that project to the paraventricular nucleus. When examined ultrastructurally, β-subunit immunoreactive terminals were found to be in synaptic contact with oxytocin-immunoreactive dendrites. Stimulation of the nucleus tractus solitarius resulted in oxytocin release into the peripheral circulation as did infusion of activin into the paraventricular nucleus. Administration of antisera against the β-chain attenuated suckling- induced oxytocin release (Roberts et al., 1989). The results from these studies suggested that activin may mediate suckling-induced and perhaps parturition-related oxytocin release.

7. Aims of thesis

The overall aim of the experiments performed in this thesis was to investigate the production and secretion of ovarian inhibin in the primate. The stumptailed macaque and common marmoset were ideal models for investigations of inhibin secretion in the primate as the hormonal profile of inhibin during the menstrual/ovulatory cycle was very similar to that of the human, with elevations in peripheral inhibin concentrations during the luteal phase. This profile is very different to the inhibin concentrations observed in sheep, cow and rat where elevations of inhibin during the luteal phase are not noted.

In chapter 2, the endocrine role of inhibin was investigated by establishing and validating an inhibin RIA for use in the stumptailed macaque and common marmoset monkey. This assay was then used to measure inhibin during the normal menstrual cycle in the macaque, the ovulatory cycle and pregnancy in the marmoset and to investigate the source and control of inhibin secretion in the human. In chapter 3, the aims were to examine the control of inhibin secretion *in vivo* using LHRH antagonist treatment, to investigate whether suppression of inhibin secretion by LHRH antagonist could be prevented by hCG or FSH and to examine the control of inhibin secretion *in vitro* using a monolayer culture system for dispersed human luteal cells. In chapter 4, the aim was to examine the effects of LHRH agonist implant in macaque, marmoset and human and following ovarian hyperstimulation in the macaque. Finally, in chapter

5, the aim was to examine the cellular localization of inhibin within the primate ovary using immunocytochemistry.

Chapter 2

The measurement of Inhibin in Primates: Stumptailed Macaque, Common Marmoset Monkey and Human.

Part I: Inhibin concentrations during the Normal Menstrual Cycle in the Stumptailed Macaque.

2.1.1 Introduction

The stumptailed macaque (*Macaca arctoides*), an Old World primate, is an excellent model for the study of reproductive endocrinology in primates. Similar to the human, these animals have menstrual cycles consisting of a follicular phase of approximately 10-14 days and a luteal phase of 14-16 days. Menstrual cycles can be monitored by hormonal measurements in daily blood samples and by recording menstrual bleeding. The important contributions to reproductive biology using the stumptailed macaque as a model are exemplified by the studies involving LHRH antagonists (described in chapter 1) which demonstrated that progesterone secretion by the corpus luteum is dependent on pituitary gonadotrophin support.

The aim of the studies described in part I was to establish and validate a radioimmunoassay for the measurement of inhibin during the normal menstrual cycle in the stumptailed macaque. The radioimmunoassay for inhibin was developed in Melbourne and at the time when these studies were carried out, there were no reports of the use of this assay in non-human primates, although unpublished observations from the Melbourne group showed that the assay was valid for use in the stumptailed macaque.

2.1.2 Materials and Methods

2.1.2.1 Animals

Eighteen adult female macaques (*Macaca arctoides*) weighing 8-13kg were used to obtain control cycles. The animals were caged singly or in pairs in rooms at a temperature of 24-26°C. The rooms were open to daylight but also lit artificially between 07.00 and 19.00h. The animals were fed a primate diet (Old World Monkey diet; B.P.Nutrition, Witham, Essex) daily and given fresh fruit five times per week, with water available *ad libitum*. All animals demonstrated regular menstrual cycles as

defined by regular elevations in progesterone concentrations >12 nmol/L for 12-14 days and regular bleeds. This was determined by examination of daily menstrual bleeding records and serum profiles of progesterone, measured in samples taken three times per week. Blood samples (4ml) were collected daily by femoral venepuncture without anaesthesia throughout the menstrual cycle and the animals were rewarded with 2ml syrup containing ferrous fumarate (Fersamal, Duncan Flockhart & Co. Ltd., Greenford, Middlesex). Blood samples were centrifuged at 1000g for 20 min and the serum stored at -20°C until assayed for LH, progesterone and inhibin.

2.1.2.2 Radioimmunoassays

Ovulation was determined by use of a rapid progesterone radioimmunoassay (RIA) together with an LH RIA.

All additions were made using an automatic dispensing system (Microlab M) into 75mm x 10mm LP3 plastic test-tubes (Sarstedt, Leicester). All assays included duplicate estimations of total binding (TC), non-specific binding (NSB), standards and samples.

(i)LH assay

^{125}I-ovine LH (iodination material: oLH LER-1056-C2) was prepared using the lactoperoxidase technique by Mrs Gwen Cowen. The antiserum (GDN-15, provided by Dr.G.Niswender) was raised in rabbits against ovine LH and was used at an initial dilution of 1:15,000 (Niswender et al., 1971). LH concentrations were expressed in terms of macaque hormone reference preparation NICHD-rhLH-RPI (1.25-320 ng/ml). The detection limit of the assay was 40µg/L and intra- and interassay coefficients of variation were 8% and 14% respectively.

LH Assay buffer

0.01M phosphate buffered saline /0.1% BSA (bovine serum albumin, RIA grade, fraction V, Sigma Chemical Co., Poole) (pH 7.4) containing:

NaCl	9g/L
Na_2HPO_4	0.35g/L
$Na_2PO_42H_20$	1.18g/L
Thiomersalate	0.1g/L

All chemicals from BDH Chemicals Ltd., Poole.

LH Assay procedure

Day 1: 100µl standard (or sample), 300µl buffer, 50µl antibody

Day 2: 50µl tracer

Day 3: 100µl each of normal rabbit serum (Scottish Antibody Production Unit, Carluke, batch no. 5263M at a dilution of 1:800) and donkey anti-rabbit serum (Scottish Antibody Production Unit, batch no. 5257M at a dilution of 1:32).

Day 4: 1ml 0.9% saline, assay was then spun at 2500 rpm for 30 min at 4°C. Supernatant was decanted and the assay then counted in a gamma counter (Pharmacia-Wallac 1261 Multigamma manual gamma counter) linked to a digital data logger (Mutek Data Grabber).

Data analysis

Data were calculated using 'Assayzap Universal Assay Calculator' developed by Dr.P.L.Taylor for Elsevier, Biosoft, U.K. This programme uses log-logit transformation of the binding data for the standards and a weighted four parameter logistic to adjust the non-specific binding (NSB), the zero value (B_O), the slope of the curve at its steepest point and the point of inflection. This minimizes the effects of outlying points in the standard curve until the optimum fit is obtained. AssayZap stores information from previous assays allowing current assays to be compared with previous assays i.e. between assay and within assay variation, quality control values and standard curves stored in a 'graveyard' can be compared between each assay. All samples above or below the assay detection limit were re-assayed at the appropriate volume.

(ii) Progesterone Assay

Progesterone concentrations were measured using a non-extraction RIA. The antiserum (361) was raised by immunizing sheep against progesterone-11α-hemisuccinate-bovine serum albumin conjugate by R.J.Scaramuzzi, using procedures similar to those reported by Scaramuzzi, Corker, Young and Baird (1974). The antiserum was used at an initial dilution of 1:10000 and had cross reactivity of <0.001% with cortisol and oestradiol-17β and <0.1% with testosterone, androstenedione and pregnenolone (Clarke, 1976). Progesterone was iodinated by Mr. Ian Swanston using the chloramine T method (Corrie et al., 1982) and purified on a 15 cm LH20 column (Amicon Ltd., Stonehouse, Gloucestershire), eluted with methanol/ethyl acetate (60:40). A progesterone 11α-glucuronide-tyramine conjugate was used for iodination and Pregn-4-ene 3, 20-dione (Sigma Chemical Co. Ltd.) (P0130) used as reference standard. The sensitivity of the progesterone assay was 0.07 pmol/tube and inter- and intra-assay coefficients of variation were 15% and 4% respectively.

Progesterone Assay Buffer
Phosphate Citrate (pH 6)
containing:

Na_2HPO_4	17.85g/L
Citric Acid Powder	7.75g/L
Thiomersalate	0.1g/L

Buffer was corrected to pH 6 and 1g/L gelatin added. All chemicals from BDH Chemicals Ltd.

Progesterone Assay procedure
Day 1. 100µl standard (or 50µl sample), 100µl antiserum (diluted 1:10,000 in assay buffer), 100µl tracer (12-15,000 cpm/tube diluted in assay buffer without gelatin, with 1mg/ml ANS (8-anilino-1-naphthalene sulphonic acid, (Sigma Chemical Co.))
Assay was then incubated for 3 hours at room temperature or overnight at 4°C.

Day 2. 100µl of donkey anti-goat serum (Scottish Antibody Production Unit, batch no. 5365N at a dilution of 1:64) and 100µl of normal sheep serum (Scottish Antibody Production Unit, batch no. 5387N at a dilution of 1:3200). Assay was then incubated overnight at 4°C.

Day 3. 1ml of 0.9% sodium chloride containing 0.2% triton X-100 and 4% polyethylene glycol (all chemicals from BDH Chemical Co., Ltd.). Assay spun at 2500 rpm for 30 minutes at 4°C and supernatant decanted. Assay then counted in a gamma counter and data analysed as per LH assay.

(iii) Inhibin Radioimmunoassay

The antiserum and hormone for iodination were purchased from Monash University, Melbourne, Australia. The antiserum (no.1989) was raised in a rabbit against 31kDa bovine inhibin using the method described by McLachlan et al. (1986a). 350µl of freeze dried antiserum was obtained from Monash and this was rehydrated with an equal volume of distilled water and stored in aliquots at 1:100 at -20°C. The hormone for iodination was prepared according to the method described by McLachlan et al. (1987a). It was redissolved in 300µl of distilled water and stored in aliquots of 20µl for labelling at -70°C.

(a) Iodination of bovine 31kD inhibin

Iodination buffers

Buffer (1) 0.5M phosphate buffer (pH 7.4)

Solution A. 0.5M $NaH_2PO_4.2H_2O$ (BDH Chemicals Ltd.) 7.8g/100ml distilled water.

Solution B. 0.5M Na_2HPO_4 anhydrous (BDH Chemicals Ltd.) 7.1g/100ml

Buffer preparation:

23.5ml solution A + 100ml solution B were mixed, adjusted to pH to 7.4 and frozen in 20ml aliquots.

Buffer (2) 0.2M phosphate buffer (pH 6.0) + 0.1% BSA

Solution A. 0.2M $NaH_2PO_4.2H_2O$ 31.2g/L

Solution B. 0.2M Na_2HPO_4 anhydrous (BDH Chemicals Ltd.) 28.39g/L

89ml solution A + 11ml solution B were mixed and made up to 100ml. 0.1g BSA was then added.

Buffer (3) 0.01M phosphate buffer (pH 7.4) + 0.1% Triton X-100

A 20ml aliquot of buffer (1) was thawed and made up to 1000ml. 0.5% BSA , 0.15M sodium chloride (BDH Chemicals Ltd.) and 0.1% Triton X-100 (Sigma Chemical Company Ltd.) was added.

Iodination method

Iodination and subsequent purification of bovine 31kD inhibin was performed as described by McLachlan et al (1986a) and Robertson et al (1988a,1988b). 40µl of buffer (1) was added to 20µl of inhibin iodination material in a 1.5ml Eppendorf tube (BDH Chemicals Ltd.). 10µl of Na ^{125}-I (1mCi) (Amersham International, Buckinghamshire)was then added and the mixture counted (TC) using a Geiger counter (type 540m with type 5-42 probe, Mini-Instruments Ltd., Essex). 80µl of 400µg/ml chloramine T (trihydrate) (BDH Chemicals Ltd.) in buffer (1) was added to start the reaction which was mixed at room temperature for 60 seconds. The reaction was terminated by adding 40µl of 3mg/ml sodium metabisulphite (BDH Chemicals Ltd.)in distilled water followed by 800µl of buffer (2).

The mixture was then applied to PD10 column (5cm; Sephadex G25, Pharmacia Ltd., Milton Keynes) previously equilibrated with 30mls of buffer (2). (The Eppendorf tube and glass pipette used for applying solution to the column were also counted and residue (R) noted). The total amount of activity applied to the column is (TC-R). The mixture was eluted using buffer (2) and 6 drops per fraction were collected using a

Figure 2. 1: Example iodination:

(all values expressed as counts per 10 sec (cp10s))

TC = 426028, R = 10225

Therefore, activity applied to column = 415803

Fraction number/Radioactivity (cp10s)

1. 121	12. 23243	23. 1794	34. 29752
2. 116	13. 30034	24. 3004	35. 26419
3. 142	14. 25467	25. 6084	36. 22871
4. 122	15. 13255	26. 8040	37. 15174
5. 137	16. 6761	27. 8073	38. 10311
6. 151	17. 3581	28. 10196	39. 7082
7. 151	18. 2488	29. 13984	40. 4540
8. 134	19. 1958	30. 19175	41. 3108
9. 220	20. 1716	31. 24487	42. 2030
10. 1185	21. 1532	32. 28964	43. 1481
11. 9435	22. 1609	33. 30498	44. 754

Figure 2.1 contd.

Pool fractions 11-18, make up to 20ml.

1ml of pool= 4421, therefore total activity in pool = 20 x 4421 = 88420

W1; 1ml = 1254, total activity in W1 = 20 x 1254 = 25080. This is 28% of the total activity applied to column. Purification procedure may therefore continue.

R1= 42305, R2 = 7081

PD1; 1ml=13269, total activity = 3 x 13269 = 39807

PD2; 1ml = 1929, total activity = 3 x 1929 = 5787

Total activity = PD1 + PD2 =45594

To calculate total amount of activity incorporated:

1mCi = 426028

45594 = 11% of 1mCi

Therefore, total activity incorporated = 110μCi

Total counts pooled from fractions 11-18=114264. This is 27% of the total activity applied to column. Since 11% was incorporated (110μCi), this leaves 16% (160μCi) solid waste and 73% (730μCi) liquid waste.

fraction collector (model 203, Anachem Ltd., Luton). All fractions were counted at 15 cm with a Geiger counter and fractions containing the iodinated peak were pooled (usually fractions 11-18) and diluted to 20 mls with buffer (2). 1ml of the diluted tracer was then counted (P) and thus the total activity was (20xP). The tracer pool was then further purified as follows:

A Red Sepharose column was prepared by applying 400µl Red Sepharose gel (Pharmacia Ltd.) to a short glass pipette which was stoppered at the end with a glass bead. The column was washed with 10mls of buffer (2). The diluted tracer was then added to the column and the wash collected (W1). 1ml of the wash was counted and the total activity noted (20xW1). If the activity was <40% of the total amount applied to the column, the purification procedure was continued. If however the activity was >40% , the wash was reapplied to the column and the activity of the eluate measured once again. The column was washed with 3x1ml of buffer (2) which was then discarded. The tracer was eluted with 2x1ml of buffer (2) containing 1M potassium chloride (BDH Chemicals Ltd.), 4M urea (BDH Chemicals Ltd., Poole) and the two fractions collected and counted (R1 and R2).

Two PD10 5cm columns (Pharmacia Ltd.) were equilibrated with 30mls of buffer (3). R1 and R2 were then applied to separate columns followed by 1.5mls of buffer (3). The eluant was discarded and a further 3mls of the wash buffer applied. A 3ml fraction from each column was collected (PD1 and PD2). 1ml from each fraction was counted and the total activity calculated [(3xPD1) + (3xPD2)]. Fractions were then pooled, aliquoted into 500µl fractions and stored at -20°C. An example iodination is shown in figure 2.1. Figure 2.2 is a representative elution profile from an inhibin iodination. Peak 1 represents labelled ^{125}I-inhibin which is eluted before 'free' ^{125} iodine (peak 2).

(b) Preparation of Inhibin Standard

The inhibin standard was prepared from partially purified human follicular fluid by Dr. Kogie Reddi (Reddi et al, 1990a). A pool of human follicular fluid was obtained from patients undergoing oocyte retrieval. The pH of the pool was adjusted to 7.2 and it was then applied to a Red Sepharose column (Pharmacia Ltd.) (Miyamoto et al, 1985). The inhibin-containing fraction was further purified by immunoaffinity chromatography using an antibody raised against a synthetic N-terminal 1-26 amino acid porcine inhibin α–subunit peptide (from Dr.Alan McNeilly). The inhibin-containing fraction was eluted in 8M urea-1M potassium chloride and dialysed against 10mM phosphate buffer. The bioactivity of the preparation was measured against the activity of ovine rete testis fluid standard (oRTF) using an *in vitro* sheep pituitary cell

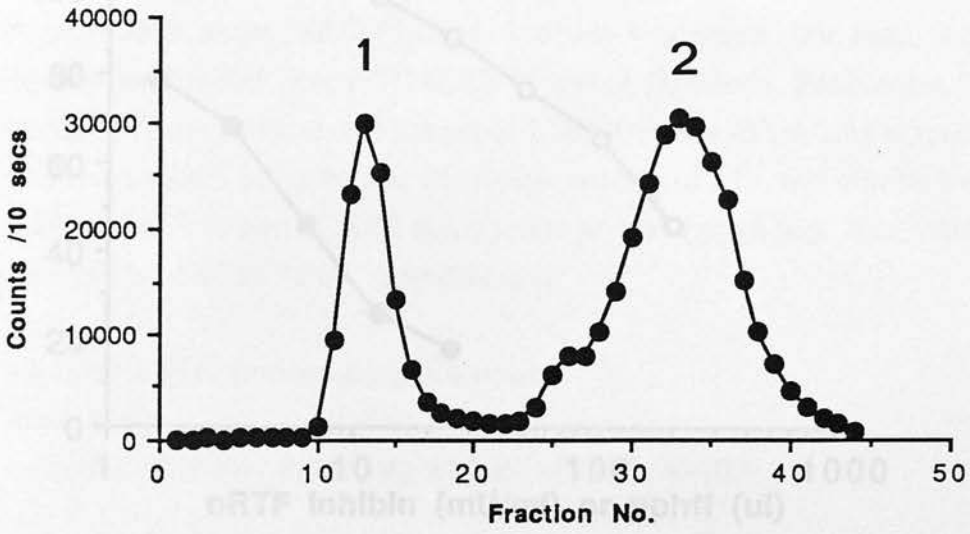


Fig.2.2. Representative elution profile from inhibin iodination- Peak 1 contains labelled ^{125} I-inhibin. Peak 2 contains free ^{125}-iodine.

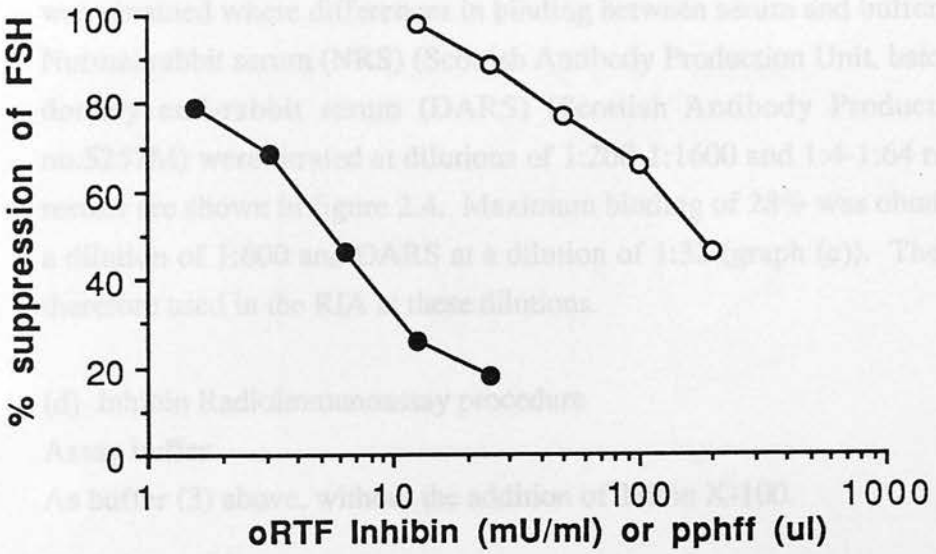


Fig. 2.3 Activity of ovine rete testis fluid (O) and partially purified human follicular fluid (●) expressed as % suppression of FSH in the sheep pituitary cell bioassay (from Dr. Kogie Reddi).

bioassay (Tsonis et al, 1986). The mean activity from seven bioassays was found to be 23.0±1.2 U/L (mean±SEM). This is shown in figure 2.3 which demonstrates the parallelism obtained between our partially purified human follicular fluid standard and the activity of ovine rete testis fluid, expressed in terms of % suppression of FSH in the sheep pituitary cell bioassay.

(c) Second Antibody Titrations

To reduce any non-specific effect of serum in the RIA, it was necessary to titrate the second antibodies used in order to obtain the concentration at which maximal binding was obtained where differences in binding between serum and buffer were negligible. Normal rabbit serum (NRS) (Scottish Antibody Production Unit, batch no.5162L) and donkey anti-rabbit serum (DARS) (Scottish Antibody Production Unit, batch no.5257M) were titrated at dilutions of 1:200-1:1600 and 1:4-1:64 respectively. The results are shown in figure 2.4. Maximum binding of 28% was obtained with NRS at a dilution of 1:600 and DARS at a dilution of 1:32 (graph (c)). The antibodies were therefore used in the RIA at these dilutions.

(d) Inhibin Radioimmunoassay procedure

Assay buffer

As buffer (3) above, without the addition of Triton X-100.

Day 1. 100µl of standard (prepared by 1:10 dilution of stock standard described in (b) and then double diluting to give standards ranging from 230-3.6 mU/0.1ml) or 100µl of serum sample, 200µl assay buffer and 100µl of antiserum (1:3000 diluted in assay buffer containing 1:600 normal rabbit serum). All samples assayed at <100µl were equalised with a pool of post-menopausal plasma, assayed previously and shown to contain undetectable amounts of inhibin. Assay incubated for 24 hours at room temperature.

Day 2. 100µl of radiolabelled tracer (±10,000 cpm per tube, diluted in buffer (3)). Assay incubated for 24 hours at room temperature.

Day 3. 100µl of donkey anti-rabbit serum (diluted 1:32 in assay buffer) was then added. Assay incubated for 24 hours at 4°C.

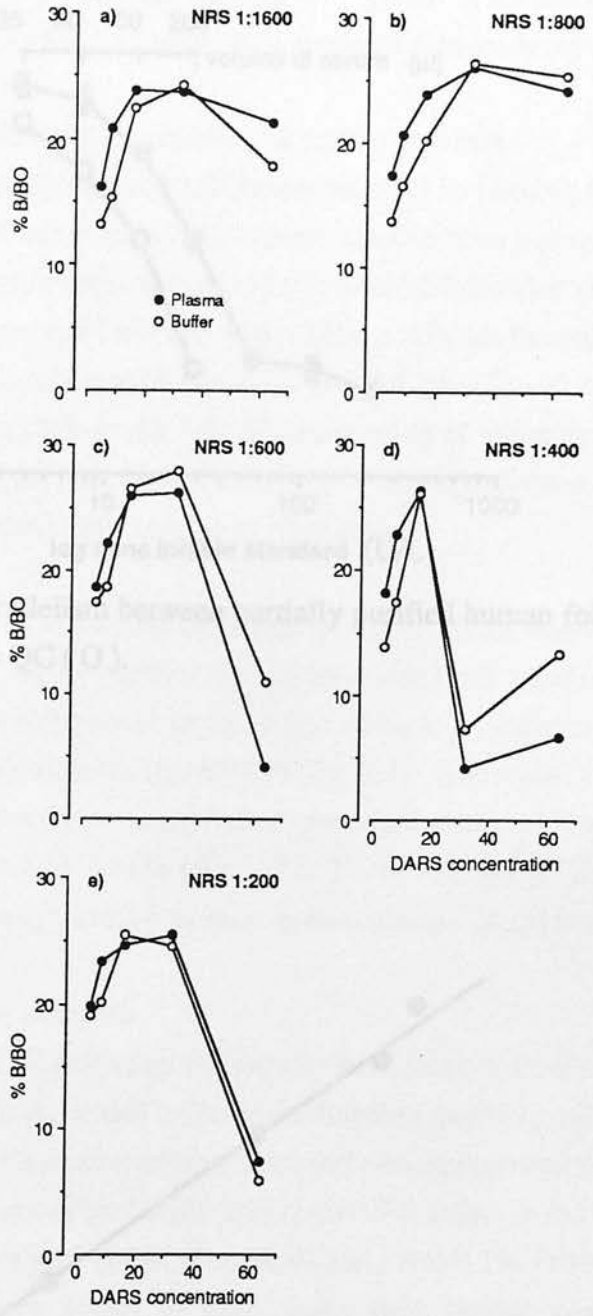


Fig.2.4 Second antibody titrations of DARS and NRS for use in inhibin radioimmunoassay (● plasma, O buffer). Maximum binding (28%) was obtained with NRS (1:600) and DARS (1:32) as shown in graph (c).

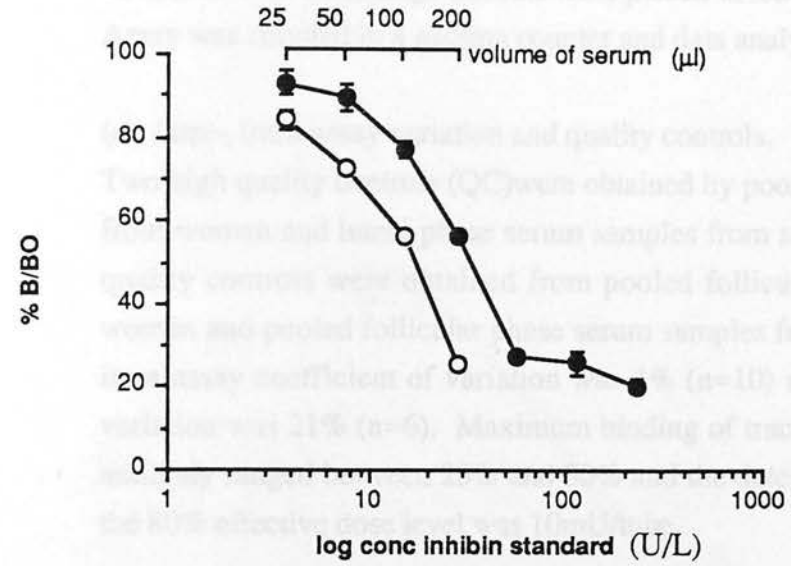


Fig. 2.5. Parallelism between partially purified human follicular fluid standard (●) and macaque QC (O).

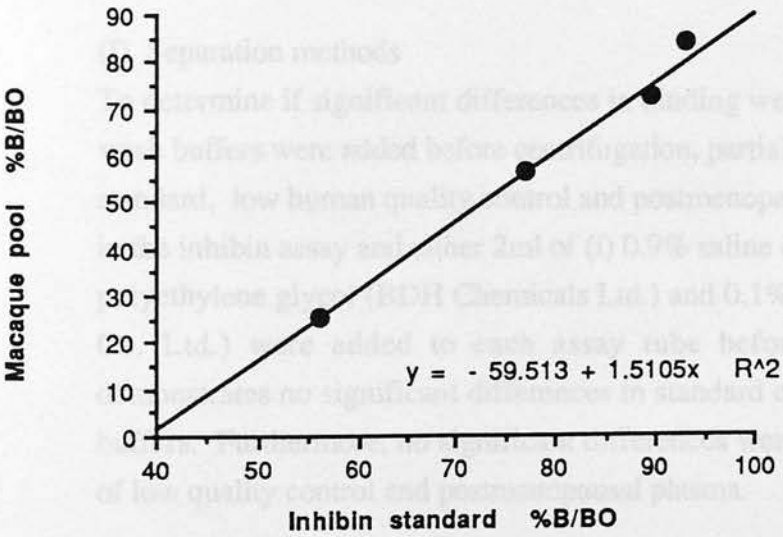


Fig. 2.6. Regression analysis between partially purified human follicular fluid standard and macaque QC.

Day 4. 2mls of 0.9% saline were added and assay spun in a centrifuge at 3000rpm for 45 min at 4°C. Centrifuge buckets were placed in ice while supernatant was aspirated. Assay was counted in a gamma counter and data analysed as per LH assay.

(e) Inter-, intra-assay variation and quality controls.

Two high quality controls (QC)were obtained by pooling luteal phase plasma samples from women and luteal phase serum samples from stumptailed macaques. Two low quality controls were obtained from pooled follicular phase plasma samples from women and pooled follicular phase serum samples from stumptailed macaques. The intra-assay coefficient of variation was 4% (n=10) and the interassay coefficient of variation was 21% (n=6). Maximum binding of tracer at the working dilution of the antibody ranged between 25% and 30% and the detection limit of the assay based on the 80% effective dose level was 10mU/tube.

Serial dilutions of the macaque high QC gave a dose-response curve parallel to that of the pooled partially purified human follicular fluid standard, as shown by an absence of significant differences in the slopes of the logit-log dose-response curves (fig. 2.5) and a regression coefficient of 0.99 (fig. 2.6). Substitution of post-menopausal serum with serum from two stump-tailed macaques castrated 2 weeks previously produced identical standard curves (fig. 2.7). However, due to the short supply of castrate macaque serum, human post-menopausal plasma (pmp) was used as a substitute.

(f) Separation methods

To determine if significant differences in binding were obtained when different assay wash buffers were added before centrifugation, partially purified human follicular fluid standard, low human quality control and postmenopausal plasma were serially diluted in the inhibin assay and either 2ml of (i) 0.9% saline or (ii) 0.9% saline containing 4% polyethylene glycol (BDH Chemicals Ltd.) and 0.1% Triton X-100 (Sigma Chemical Co. Ltd.) were added to each assay tube before centrifugation. Figure 2.8 demonstrates no significant differences in standard curves were obtained using wash buffers. Furthermore, no significant differences were obtained in the serial dilutions of low quality control and postmenopausal plasma.

(g) Comparison of plasma and serum in assay

To compare if differences in binding were obtained between plasma and serum samples, daily blood samples (4ml) were collected by femoral venepuncture without anaesthesia from two macaques (animal housing and sample collection as previously

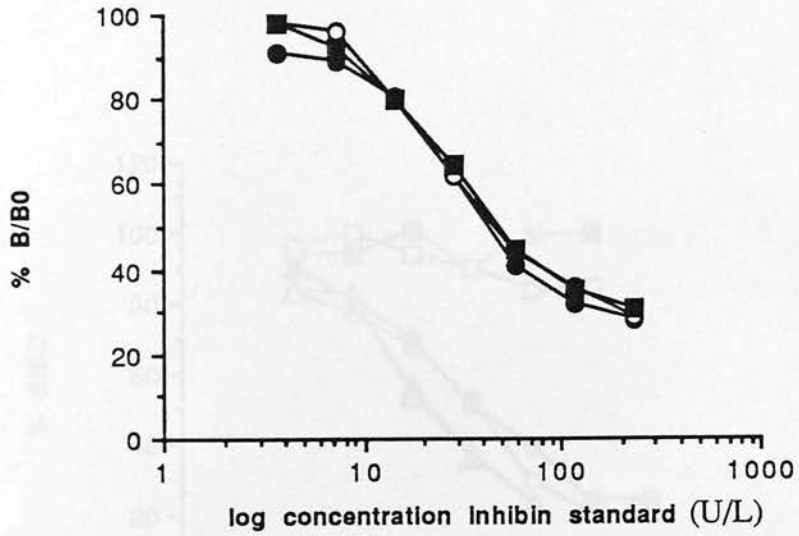


Fig. 2.7. Inhibin standard curves containing post-menopausal plasma (●), castrate macaque serum no.12 (○) and castrate macaque serum no. 16 (■).

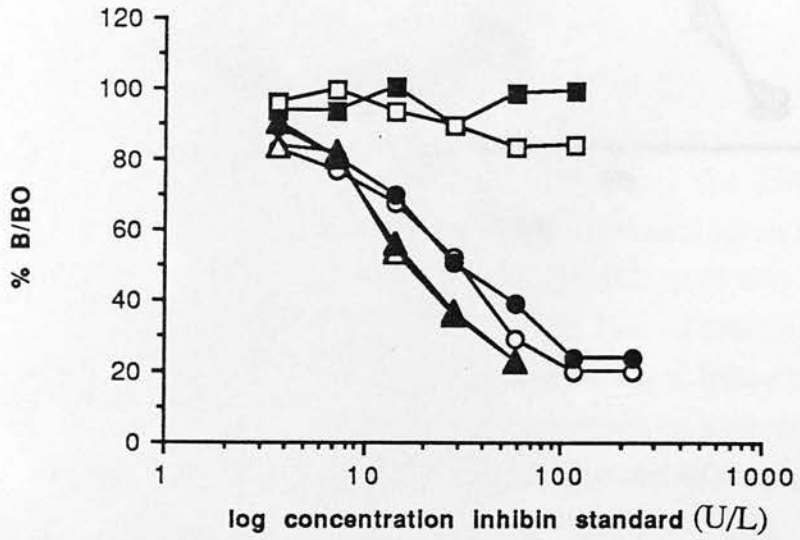


Fig. 2.8. Comparison of % binding in inhibin RIA using saline or polyethylene glycol (PEG) as a wash buffer before centrifugation: pphff separated using saline (●) and PEG (○), post-menopausal plasma separated using saline (■) and PEG (□) and low QC separated using saline (▲) and PEG (△).

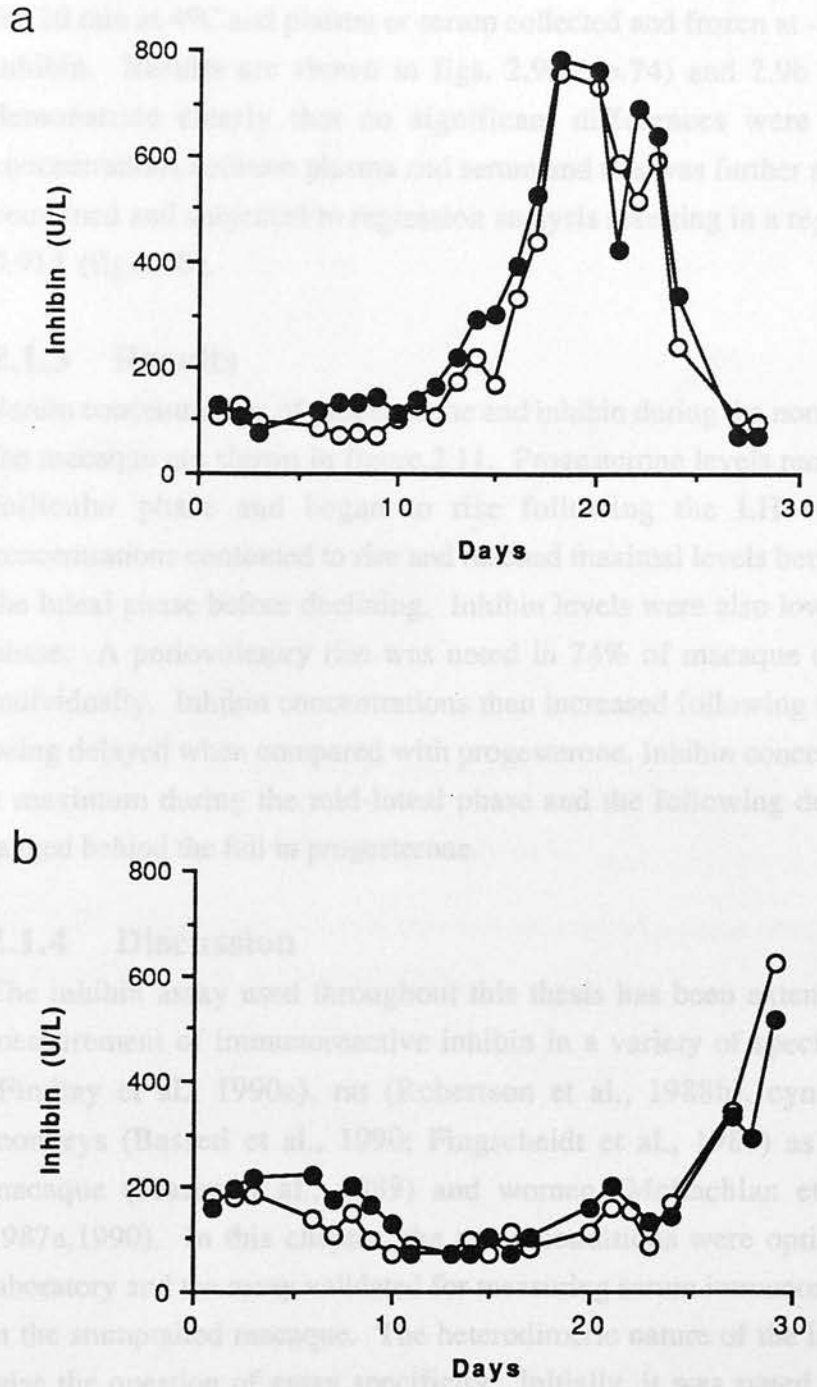


Fig. 2.9. Comparison of serum (●) and plasma (O) inhibin concentrations throughout the menstrual cycle in macaque 74 (a) and 75 (b).

described in chapter). Blood samples (2ml) were placed in a lithium heparin blood tube for plasma collection and the remaining sample into a 2ml plastic sample tube and left at room temperature to clot for 3h for serum collection. Samples were spun at 2500g for 20 min at 4°C and plasma or serum collected and frozen at -20°C until assayed for inhibin. Results are shown in figs. 2.9a (no.74) and 2.9b (no.75). These data demonstrate clearly that no significant differences were obtained in inhibin concentrations between plasma and serum and this was further shown when data were combined and subjected to regression analysis resulting in a regression coefficient of 0.912 (fig.2.10).

2.1.3 Results

Serum concentrations of progesterone and inhibin during the normal menstrual cycle in the macaque are shown in figure.2.11. Progesterone levels remained low during the follicular phase and began to rise following the LH surge. Progesterone concentrations continued to rise and reached maximal levels between days 8 and 12 of the luteal phase before declining. Inhibin levels were also low during the follicular phase. A periovulatory rise was noted in 74% of macaque cycles when assessed individually. Inhibin concentrations then increased following the LH surge, the rise being delayed when compared with progesterone. Inhibin concentrations also reached a maximum during the mid-luteal phase and the following decline in inhibin then lagged behind the fall in progesterone.

2.1.4 Discussion

The inhibin assay used throughout this thesis has been extensively utilized in the measurement of immunoreactive inhibin in a variety of species such as the sheep (Findlay et al., 1990a), rat (Robertson et al., 1988b), cynomolgus and rhesus monkeys (Basseti et al., 1990; Fingscheidt et al., 1989) as well as stumptailed macaque (Fraser et al., 1989) and women (McLachlan et al., 1986b, 1986c, 1987a,1990). In this chapter, the assay conditions were optimised for use in our laboratory and the assay validated for measuring serum immunoreactive inhibin levels in the stumptailed macaque. The heterodimeric nature of the inhibin molecule may raise the question of assay specificity. Initially, it was stated that inhibin subunits obtained after reductive alkylation as well as other inhibin-related proteins show limited cross-reaction in this assay (Robertson et al., 1988b). However, it has since been found that an α subunit precursor (pro-αC), isolated from bovine follicular fluid, cross reacts >100% in the assay. Pro-αC consists of the pro region of the α-subunit, disulphide- linked to the mature α-subunit (Robertson et al., 1989) (fig. 1.2).

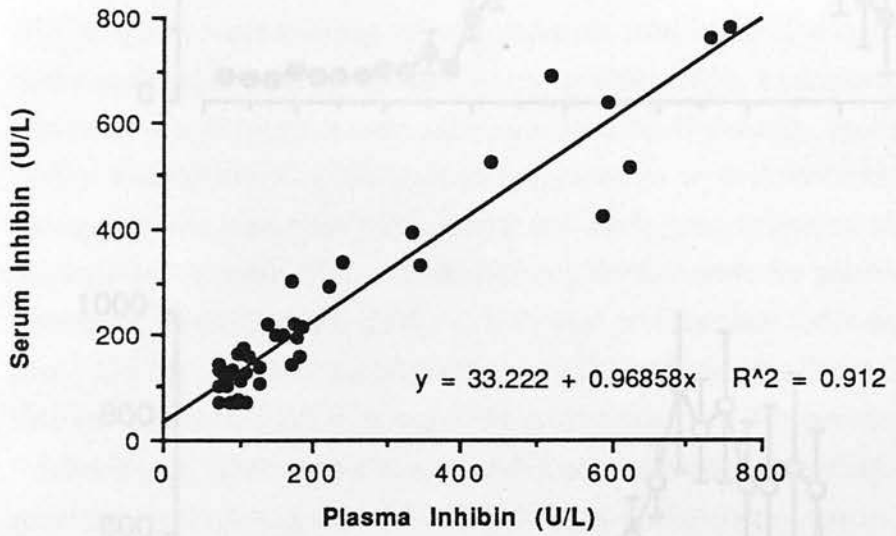


Fig. 2.10. Regression analysis between serum and plasma concentrations throughout the menstrual cycle in two macaques.

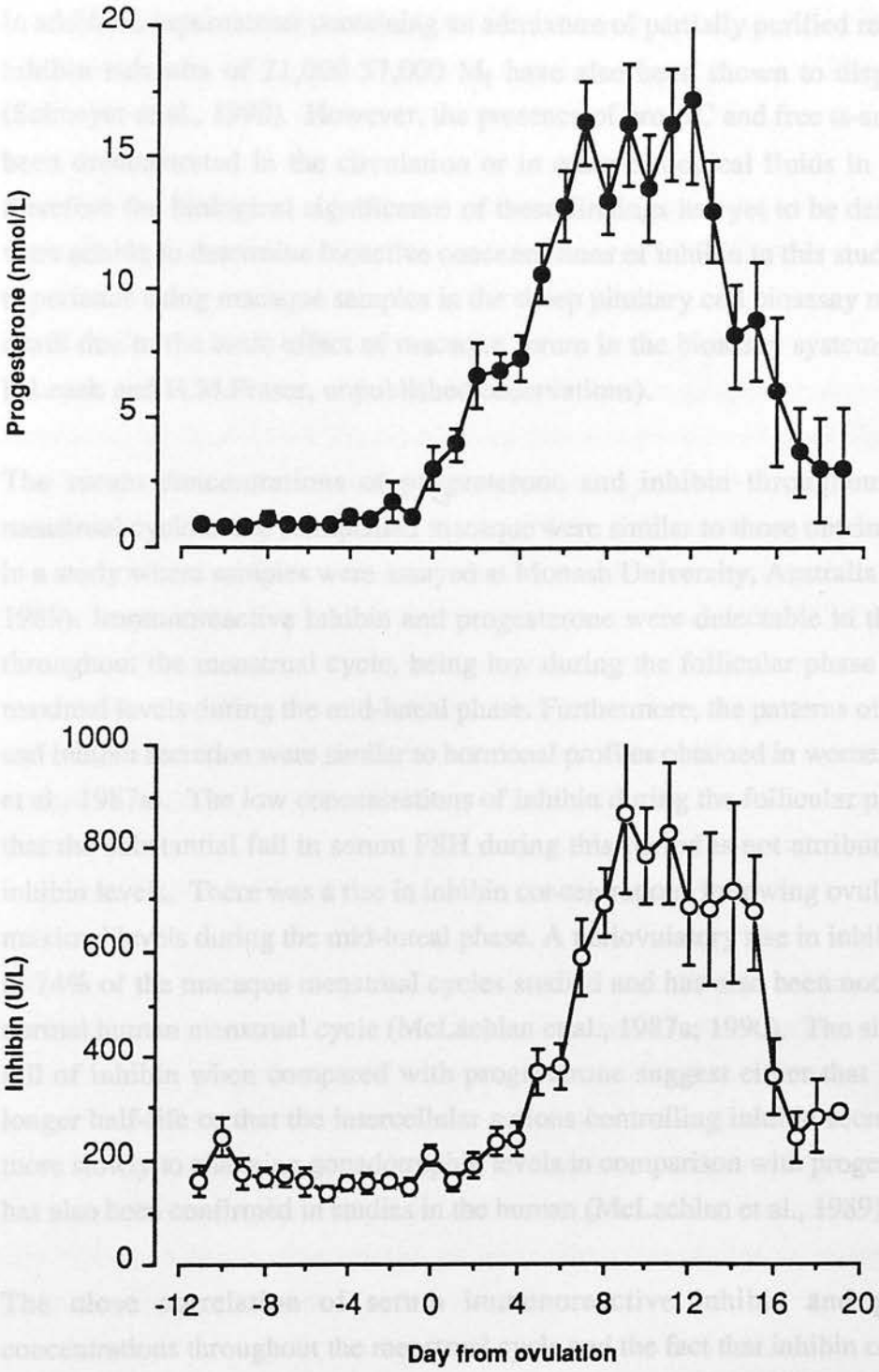


Fig. 2.11. Progesterone (●) and inhibin (O) concentrations throughout the menstrual cycle in the macaque (n=18). Values are means ± S.E.M.

In addition, supernatants containing an admixture of partially purified recombinant α-inhibin subunits of 21,000-57,000 M_r have also been shown to displace binding (Schneyer et al., 1990). However, the presence of pro-αC and free α-subunit has not been demonstrated in the circulation or in other biological fluids in primates and therefore the biological significance of these findings has yet to be determined. We were unable to determine bioactive concentrations of inhibin in this study as previous experience using macaque samples in the sheep pituitary cell bioassay resulted in cell death due to the toxic effect of macaque serum in the bioassay system (C.G.Tsonis, R.Leask and H.M.Fraser, unpublished observations).

The serum concentrations of progesterone and inhibin throughout the normal menstrual cycle in the stumptailed macaque were similar to those obtained previously in a study where samples were assayed at Monash University, Australia (Fraser et al., 1989). Immunoreactive inhibin and progesterone were detectable in the circulation throughout the menstrual cycle, being low during the follicular phase and reaching maximal levels during the mid-luteal phase. Furthermore, the patterns of progesterone and inhibin secretion were similar to hormonal profiles obtained in women (McLachlan et al., 1987a). The low concentrations of inhibin during the follicular phase suggests that the substantial fall in serum FSH during this period is not attributable to rising inhibin levels. There was a rise in inhibin concentrations following ovulation to reach maximal levels during the mid-luteal phase. A periovulatory rise in inhibin was noted in 74% of the macaque menstrual cycles studied and has also been noted during the normal human menstrual cycle (McLachlan et al., 1987a; 1990). The slower rise and fall of inhibin when compared with progesterone suggest either that inhibin has a longer half-life or that the intercellular actions controlling inhibin secretion respond more slowly to changing gonadotrophin levels in comparison with progesterone. This has also been confirmed in studies in the human (McLachlan et al., 1989).

The close correlation of serum immunoreactive inhibin and progesterone concentrations throughout the menstrual cycle and the fact that inhibin concentrations reached a maximum during the mid-luteal phase suggests that the corpus luteum is a major source of inhibin during the primate menstrual cycle. mRNA for inhibin α-subunit has been identified in RNA isolated from luteal tissue in women (Davis et al., 1987b), cynomolgus monkey (Basseti et al., 1990) and marmoset monkey (Hillier et al., 1989). In addition, evidence for both α and βA subunit gene expression using *in situ* hybridization has recently been demonstrated in the primate corpus luteum (Schwall et al., 1990). Furthermore, Basseti et al., (1990) demonstrated luteectomy

during the mid-luteal phase in cynomologus monkeys resulted in a suppression of serum inhibin by 24h, providing further evidence that the corpus luteum is a major source of inhibin in the primate.

Part II: Inhibin Concentrations during the Ovulatory Cycle and Pregnancy in the Common Marmoset Monkey.

2.2.1 Introduction

The common marmoset monkey, *Callithrix jacchus* , is an appropriate model to study reproductive endocrinology in New World Primates because of its availability, ease of use, and the fact that its basic reproductive biology is well documented and shows many similarities to that of man (Hearn, 1983). Measurement of plasma hormone concentrations has shown that the marmoset has an ovarian cycle of 28-30 days, comprising a follicular phase of 8-9 days and a luteal phase of 19-22 days (Harding *et al.* 1982; Harlow et al., 1983). Following conception, implantation commences on day 12 after ovulation (Moore et al, 1985), and the young, normally twins, are delivered after a gestation period of 144 days (Chambers & Hearn, 1979). The corpus luteum is necessary to maintain the pregnancy for the first 6-9 weeks of gestation (Hearn, 1978; 1983) after which time progesterone is secreted in sufficient quantity from the feto-placental unit (Hodges et al., 1983).

The aims of the following studies in Part II were firstly, to determine plasma concentrations of immunoreactive inhibin in the common marmoset monkey during the ovarian cycle, pregnancy and in anovular and ovariectomized states; secondly, to compare these results with values obtained in the adult female macaque and human using the same heterologous radioimmunoassay and finally, in collaboration with Dr. Georgina Webley, a pilot study was performed to examine the effect of administration of a $PGF_{2\alpha}$ analogue, cloprostenol, on immunoreactive inhibin secretion during early pregnancy in the marmoset.

2.2.2 Materials and Methods

(i) Marmosets (*Callithrix jacchus*)

Adult marmosets weighing 350-400g were selected. Animals were housed in rooms maintained at temperatures between 20°C and 25°C. Rooms were artificially lit between 07.00 and 19.00. Animals were fed on 'Mazuri' New World Diet (Scientific Diet Services Ltd., Stepfield, Essex), fresh fruit, vegetables and seeds daily with water available *ad libitum*. Blood samples (300µl) were drawn from the upper region

of the femoral vein using a 27 gauge needle and a 1ml heparinised syringe while the animals were held in a restraining device (Hearn et al., 1978). This device allows the animals to be held securely without discomfort. All blood samples were taken by Dr. Thillai Koothan and the technical staff at the primate unit.

(a) Normal Cycles

Blood samples (0.5ml) were collected twice per week for a 4 month period in six adult female animals. The overall pattern of change in progesterone plasma concentrations was examined, and those animals in which there were regular increases in plasma progesterone to concentrations greater than 60nmol/L at approximately 30-day intervals were considered to be representative of animals with ovulatory ovarian cycles (n=5).

(b) Pregnancy

Six marmosets with normal cycles based on increases in progesterone as defined above were housed with male marmosets of proven fertility. Blood samples were collected twice per week until day 70 of pregnancy and were continued at approximately 2-weekly intervals from days 68-138 of pregnancy in individual animals. Sampling was reduced during the second half of pregnancy to avoid undue stress to the mother. The stage of gestation was retrospectively dated from the day of delivery, which was assumed to be full-term (144 days) on the basis of the appearance of the young.

(c) Ovariectomized and acyclic marmosets.

The concentration of inhibin obtained in terminal blood samples from four female marmosets which had been ovariectomized for at least 12 weeks was measured. A plasma pool was obtained from samples collected in four intact adult female animals in which the progesterone concentration remained undetectable throughout a period of > 10 weeks, indicating a failure of ovarian cyclicity.

(ii) Macaques (*Macaca arctoides*)

Serum samples were collected daily from five stumptailed macaques throughout a normal ovulatory menstrual cycle, such cycles being defined on the basis of serum concentrations of progesterone as described in Part I. All blood samples were centrifuged at 1000g for 30 mins and serum was stored at -20°C until assayed for progesterone and inhibin.

(iii) Human samples

Blood samples were collected daily from 5 normal women by Dr. Kogie Reddi for one complete menstrual cycle, subject to the criteria detailed in Part III. Blood samples were centrifuged at 1000g for 30 min and plasma stored at -20°C until assayed for progesterone and inhibin.

Pooled data

For the purpose of comparison between species, the marmoset data were pooled according to the stages of cycle as follows: Data obtained from plasma samples collected from the five adult female marmosets bled twice weekly for 4 months were pooled according to stage of the ovarian cycle on the basis of plasma progesterone concentrations. The follicular phase was taken as that period during which progesterone concentrations were below 60nmol/L. This limit was derived from observations by Chambers & Hearn (1979) and Harding et al., (1982) who selected a concentration of 30 nmol/L of progesterone as indicative of ovulation and conception. In the current study, to ensure that animals were indeed in the luteal phase this limit was increased to 60 nmol/L. As a result of the bleeding frequency, progesterone levels were normally below 60 nmol/L for only two or three consecutive samples; the first of these was taken as representative of the early follicular phase, the second or third as the late follicular period. The early luteal phase samples were those in which plasma progesterone had started to rise above the 60nmol/L value, and the late luteal phase comprised samples in which progesterone had shown a fall to levels just above those seen in the follicular phase. Mid-luteal phase values were taken at a stage intermediate between the early and late luteal phases, when progesterone was increased and in a plateau phase. The mean value for progesterone and inhibin at these stages was calculated from the means obtained from the cycles of each of the five animals. All samples in this portion of the study were measured in three separate inhibin assays. Macaque data were pooled to allow direct comparison according to stage of the menstrual cycle. Day 1 of the follicular phase was taken as the day on which serum progesterone concentrations fell to follicular phase values and the results pooled as follows: days 1-5 (early follicular), days 6-10 (late follicular), post-ovulation days 1-5 (early luteal), days 6-10 (mid luteal) and days 11-15 (late luteal). Human data were pooled using the same criteria.

Assays

(i) Progesterone assay

Progesterone concentrations were measured as described in Part I.

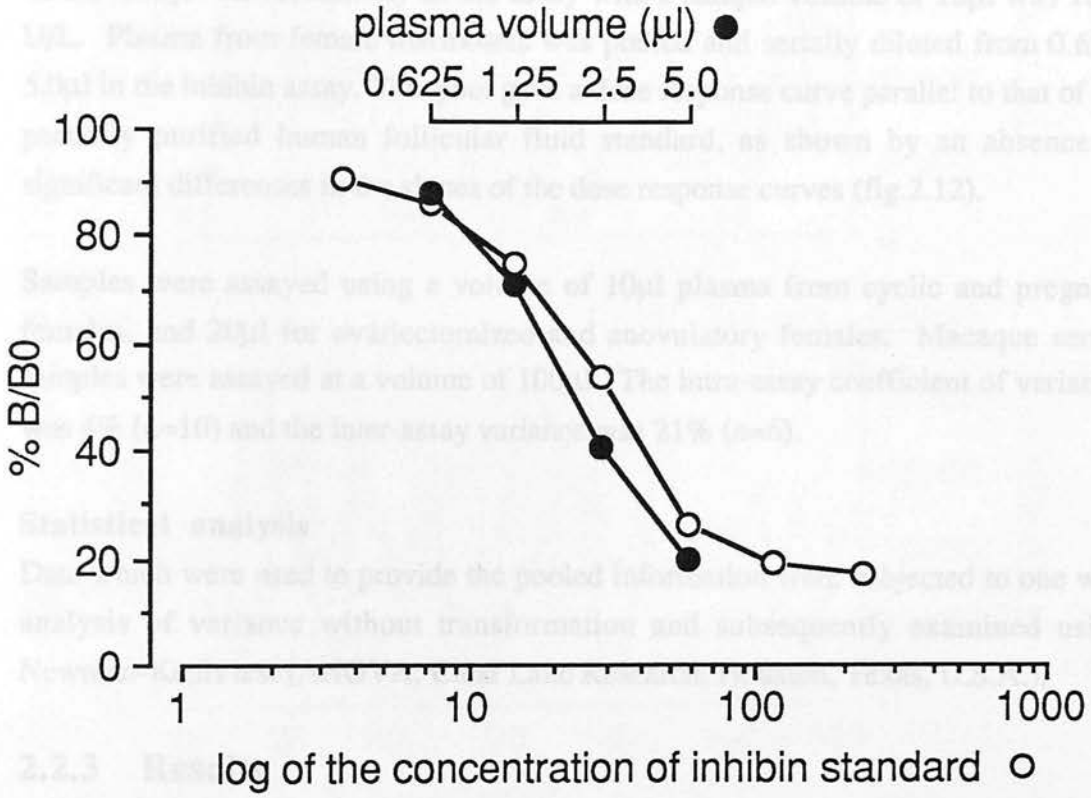


Fig. 2.12. Serial dilutions of a pool of marmoset plasma (●) demonstrate parallelism with the partially purified human follicular fluid standard (O).

(ii) Inhibin assay

Inhibin concentrations were measured using the heterologous RIA as described in Part I. Initial studies were carried out to validate the assay for measurement of inhibin in the marmoset. A pool of marmoset plasma from the luteal phase was assayed at 200-2.5µl. For this pool, volumes >10µl gave values greater than the upper detection limit of the assay. The sensitivity of the assay with a sample volume of 10µl was 1000 U/L. Plasma from female marmosets was pooled and serially diluted from 0.625-5.0µl in the inhibin assay. This pool gave a dose response curve parallel to that of the partially purified human follicular fluid standard, as shown by an absence of significant differences in the slopes of the dose response curves (fig.2.12).

Samples were assayed using a volume of 10µl plasma from cyclic and pregnant females, and 20µl for ovariectomized and anovulatory females. Macaque serum samples were assayed at a volume of 100µl. The intra-assay coefficient of variation was 4% (n=10) and the inter-assay variance was 21% (n=6).

Statistical analysis

Data which were used to provide the pooled information were subjected to one way analysis of variance without transformation and subsequently examined using Newman-Keuls test (ANOVA; Clear Lake Research, Houston, Texas, U.S.A.).

2.2.3 Results

Plasma concentrations of inhibin and progesterone during four sequential cycles from each of two representative marmosets are shown in figure 2.13. All five animals studied in this group demonstrated increases in inhibin during the luteal phase of the cycle. When the data were pooled according to stage of the cycle determined from plasma progesterone concentrations (fig. 2.14), it was evident that progesterone rose significantly ($p<0.01$) during the early-, mid- and late- luteal phase in comparison with the follicular phase while inhibin was significantly elevated ($p<0.01$) during the mid and late luteal phase.

Figure 2.14 also illustrates the inter-species comparison of inhibin and progesterone concentrations at different stages of the cycle in the marmoset, stumptailed macaque and human. The hormonal patterns were similar in the 3 species; however, the mean progesterone concentration during the follicular phase in the marmoset was approximately 35 times that of the macaque and 23 times that of the human during the same period. During the luteal phase, progesterone concentrations in the marmoset

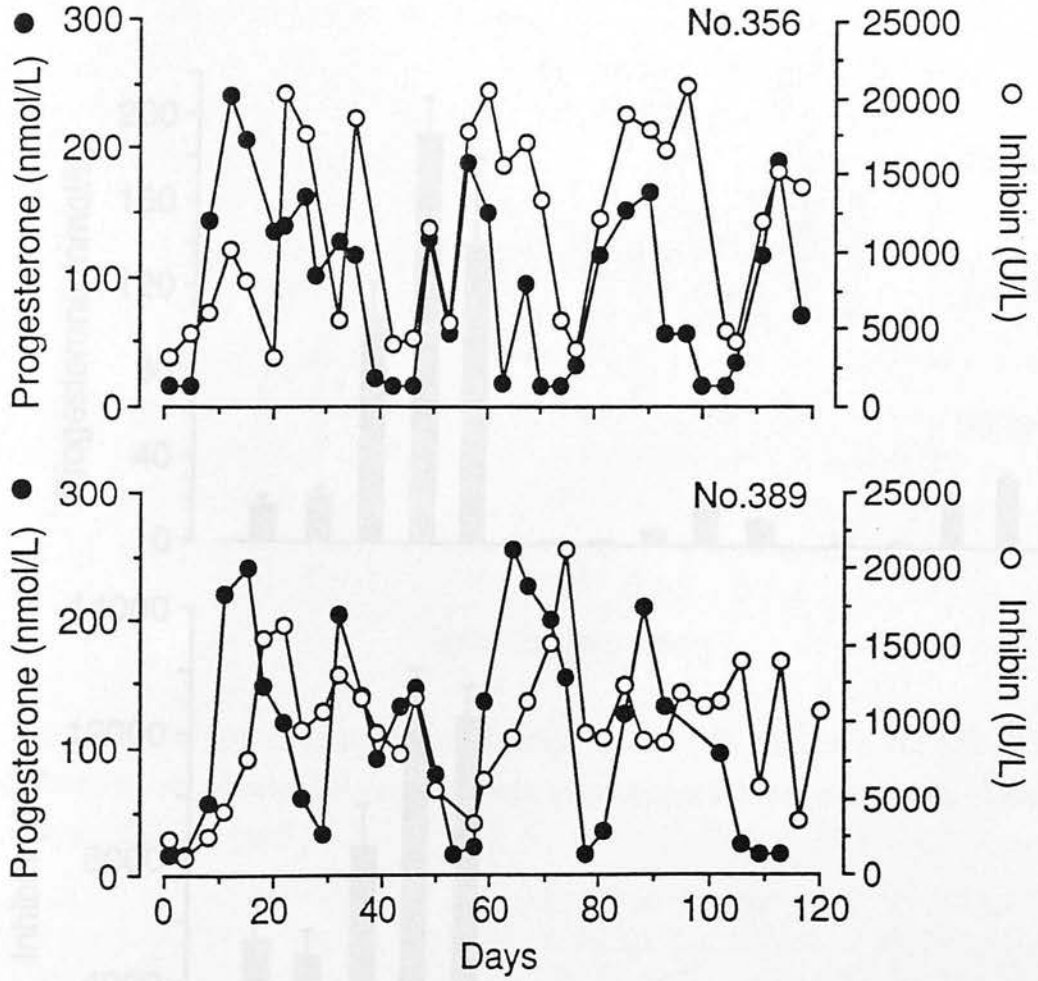


Fig. 2.13. Plasma concentrations of progesterone (●) and inhibin (O) in four consecutive cycles from two female marmosets. The luteal phase is defined as that period during which progesterone concentrations were >60 nmol/L for at least 14 days.

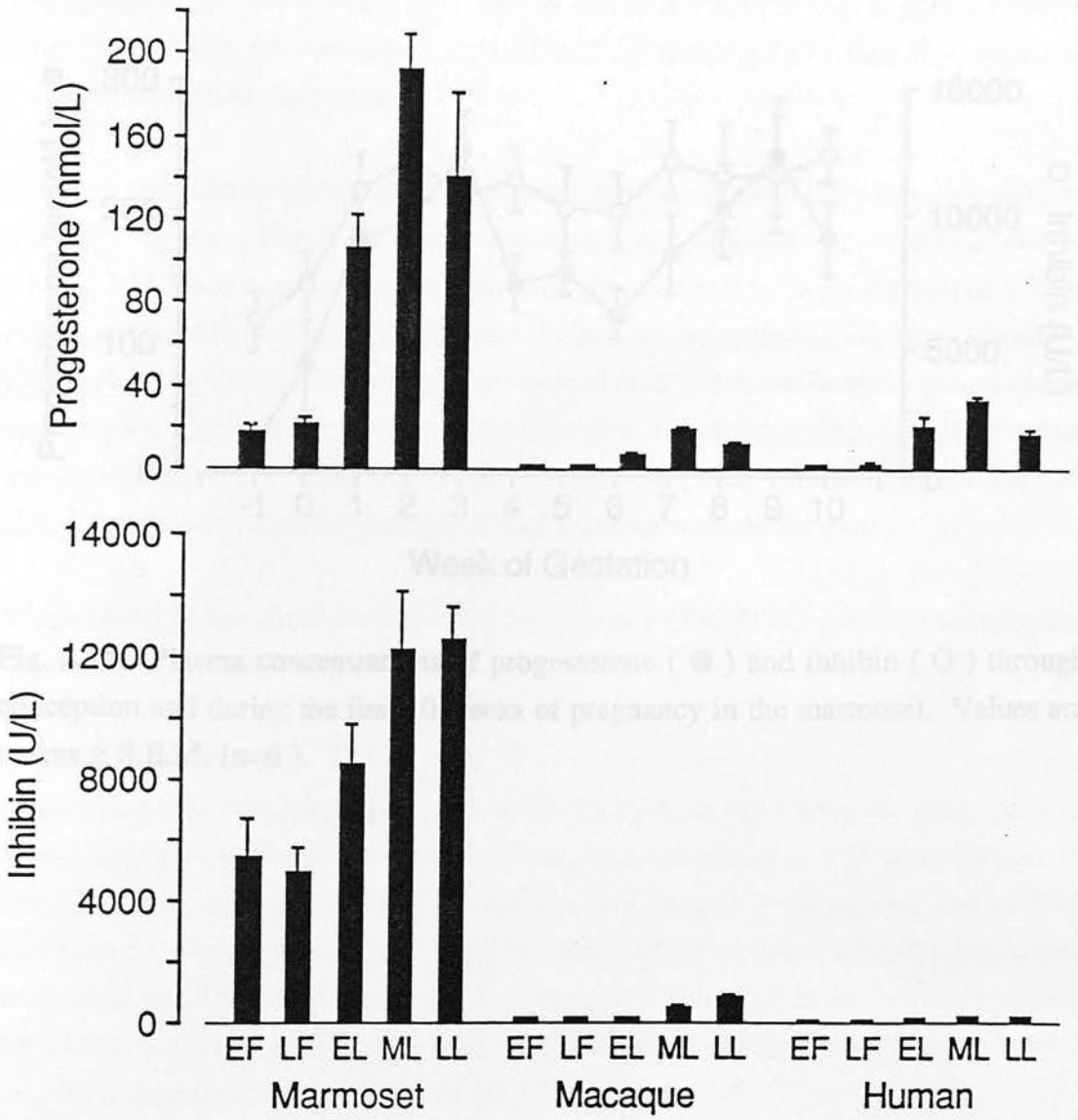


Fig. 2.14. Comparison between progesterone and inhibin concentrations in plasma in the marmoset, macaque and human during the early follicular (EF), late follicular (LF), early luteal (EL), mid-luteal (ML) and late luteal (LL) phases of the cycle. Values are means ± S.E.M. (n=5 per group).

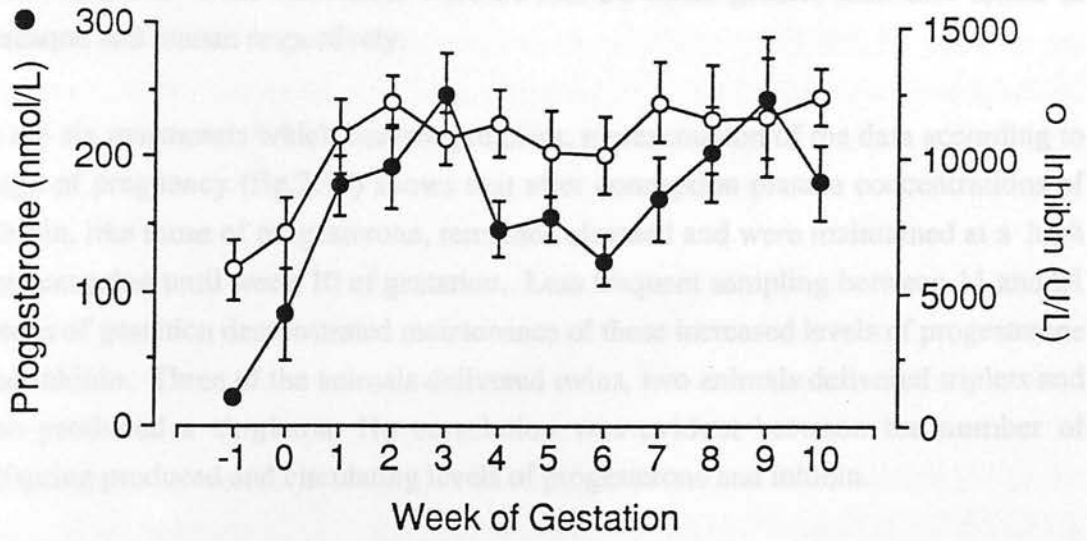


Fig. 2.15. Plasma concentrations of progesterone (●) and inhibin (O) through conception and during the first 10 weeks of pregnancy in the marmoset. Values are means ± S.E.M. (n=6).

were 14 times that of the macaque and 6 times that of the human. The differences in inhibin concentrations between species were even more pronounced. During the follicular phase, inhibin concentrations in marmoset were 46 times greater than the macaque and 89 times that in the human while during the luteal phase, inhibin concentrations in the marmoset were 28 and 56 times greater than that found in macaque and human respectively.

In the six marmosets which became pregnant, representation of the data according to stage of pregnancy (fig.2.15) shows that after conception plasma concentrations of inhibin, like those of progesterone, remained elevated and were maintained at a high concentration until week 10 of gestation. Less frequent sampling between 11 and 21 weeks of gestation demonstrated maintenance of these increased levels of progesterone and inhibin. Three of the animals delivered twins, two animals delivered triplets and one produced a singleton. No correlation was evident between the number of offspring produced and circulating levels of progesterone and inhibin.

Values were below the detection limit of the assay (<1000 U/L) in anovulatory and ovariectomized marmosets.

2.2.4 Discussion

The inhibin concentrations during the ovulatory cycle in the marmoset were found to follow a pattern similar to that observed in man (McLachlan *et al.* 1987a) and macaque (Part I). The relationship between the levels of plasma progesterone and inhibin indicates that the corpus luteum is also the source of both hormones in the marmoset. Marmoset luteal tissue also produces immunoreactive inhibin *in vitro* and expresses an inhibin α-subunit mRNA of ~ 1.5kb, suggesting that the corpus luteum is a source of immunoreactive inhibin in this species (Hillier *et al.* 1989).

This situation in these species contrasts with that in non-primate species such as the cow (Hasegawa *et al.* 1987), sheep (McNeilly *et al.* 1989), pig (Hasegawa *et al.* 1988) and rat (Taya *et al.* 1989; Rivier *et al.* 1989) in which an elevation in circulating inhibin during the luteal phase was not observed. Furthermore, α-inhibin mRNA was undetectable in corpora lutea of cattle and sheep by Northern Blot analysis or *in situ* hybridization, (Rodgers et al., 1989; Torney et al., 1989) and although α-inhibin mRNA was demonstrated in the rat corpus luteum, it was expressed at low levels, with the greatest expression in the follicle of this species (Davis *et al.*1986b: Woodruff *et al.* 1987; Meunier *et al.* 1988a).

An interesting finding in the present study was the high plasma concentration of inhibin during the ovarian cycle in the marmoset in comparison with those in the macaque and human. While part of the explanation may be attributed to the fact that the marmoset normally produces two or three corpora lutea as opposed to one in the macaque and human it would appear that the marmoset tissue is particularly active in producing inhibin. Also, inhibin concentrations during the follicular phase in the marmoset were clearly above those observed in anovulatory or ovariectomized marmosets, indicating that inhibin produced by ovarian follicles is secreted into the peripheral circulation in significant quantities in the marmoset.

Plasma concentrations of progesterone were also high in the marmoset in comparison with Old World primates and man (Lipsett *et al.*1985). In the marmoset, progesterone concentrations can rise to in excess of 300nmol/L in the luteal phase (Harding, *et al.* 1982; Harlow et al., 1984); comparable luteal phase values are 15-45 nmol/L in the rhesus macaque (Bosu et al., 1972; Elvidge & Roper, 1977), 5-45nmol/L in the stumptailed macaque as shown in the present study and by Fraser et al. 1986 and 10-65nmol/L in man (Abraham et al., 1972). These increased levels of progesterone in the marmoset are associated with the presence of a cortisol-binding globulin of very low capacity and increased plasma cortisol concentrations; thus the majority of the circulating progesterone is in the free form. As a result of these increased plasma steroid concentrations, there is a compensatory end-organ steroid resistance. Although the uterine cytosolic progesterone receptor has similar affinity to that in the Old World primate, it is present in concentrations four- to eight- fold less. A similar reduction is seen in the number of pituitary progesterone receptors (for review see Lipsett *et al.*1985). The relevance of high plasma concentrations of inhibin in the marmoset and its interrelationship with target tissue receptors remains to be investigated.

Although the marmoset is not the ideal model for pregnancy in man since, for example, there are differences in steroid metabolism (Shackleton, 1974; Shackleton & Mitchell, 1975), and in the pattern of chorionic gonadotrophin excretion (Hobson et al., 1977) between the two species, the former has the advantage of being one of the most fecund primates studied to date. The observation of a sustained plateau in inhibin during pregnancy in the marmoset is similar to that described in women (McLachlan *et al*, 1988). In addition to the corpus luteum, the human placenta has also been shown to produce inhibin (McLachlan *et al.* 1986b; Davis *et al.* 1987b). However, this study does not allow differentiation between the contribution of the corpora lutea of pregnancy and the early embryo or decidua in inhibin production.

2.2.5 Effects of Cloprostenol during early pregnancy in the common marmoset monkey.

In collaboration with Dr. Georgina Webley from the Institute of Zoology, Regent's Park, London, a preliminary study was carried out to investigate the acute and chronic effects of administration of a $PGF_{2\alpha}$ analogue, cloprostenol, on immunoreactive inhibin concentrations during early pregnancy in the common marmoset.

(i) Chronic effects. Blood samples from two pregnant marmosets were taken approximately every two days from days 12-31 after ovulation. 0.5μg cloprostenol (Estrumate: I.C.I. Macclesfield, U.K.: lot nos. HS12, P411) was administered i.m. on day 26 after ovulation.

(ii) Acute effects. Four marmosets received 0.5μg cloprostenol via i.m.injection on days 17-20 after ovulation. Blood samples were taken 2 min before and 0.5h, 1h, 1.5h, 2h and 4h after cloprostenol. Blood samples were taken from 3 control animals at the same time intervals as the cloprostenol-treated animals.

The experimental procedure and progesterone assays were carried out at Regent's Park and the plasma samples were sent to Edinburgh in dry ice for the measurement of immunoreactive inhibin using the RIA described in 2.2.2.

2.2.6 Results

Plasma inhibin and progesterone concentrations in the animals receiving cloprostenol on day 26 after ovulation are shown in figures 2.16a and 2.16b. Inhibin and progesterone concentrations remain elevated at luteal phase concentrations until administration of cloprostenol. Plasma levels of inhibin and progesterone then fell rapidly, reaching preovulatory levels by the following sampling period. Figure 2.17a and 2.17b represent the mean levels of progesterone and inhibin in control (n=3) and treated (n=4) animals in the acute group. Following administration of cloprostenol, there was a marked fall in both progesterone and inhibin concentrations by 30min and 60min respectively. Inhibin concentrations fell to less than half the initial concentration by 90min whereas progesterone concentrations were halved after 1h.

2.2.7 Discussion

The findings from this study provide further evidence that the corpus luteum is a major source of immunoreactive inhibin in the common marmoset. The rapid decline in both progesterone and inhibin concentrations following administration of cloprostenol suggests that these hormones are under similar control mechanisms in the marmoset. Previous studies using a LHRH antagonist in the marmoset (Hodges et al., 1988)

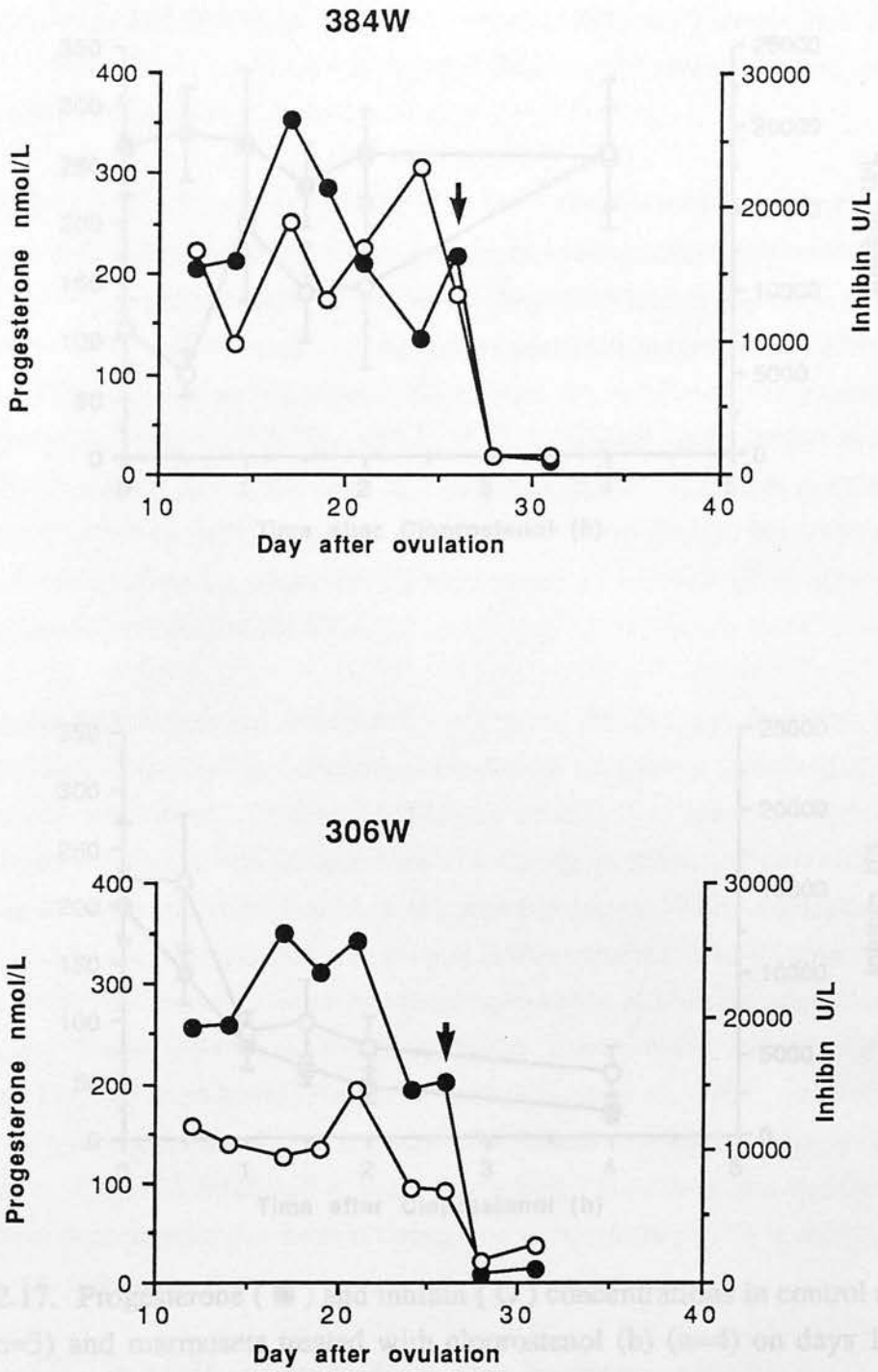


Fig. 2.16. Progesterone (●) and inhibin (O) concentrations in two pregnant marmosets receiving cloprostenol (arrows).

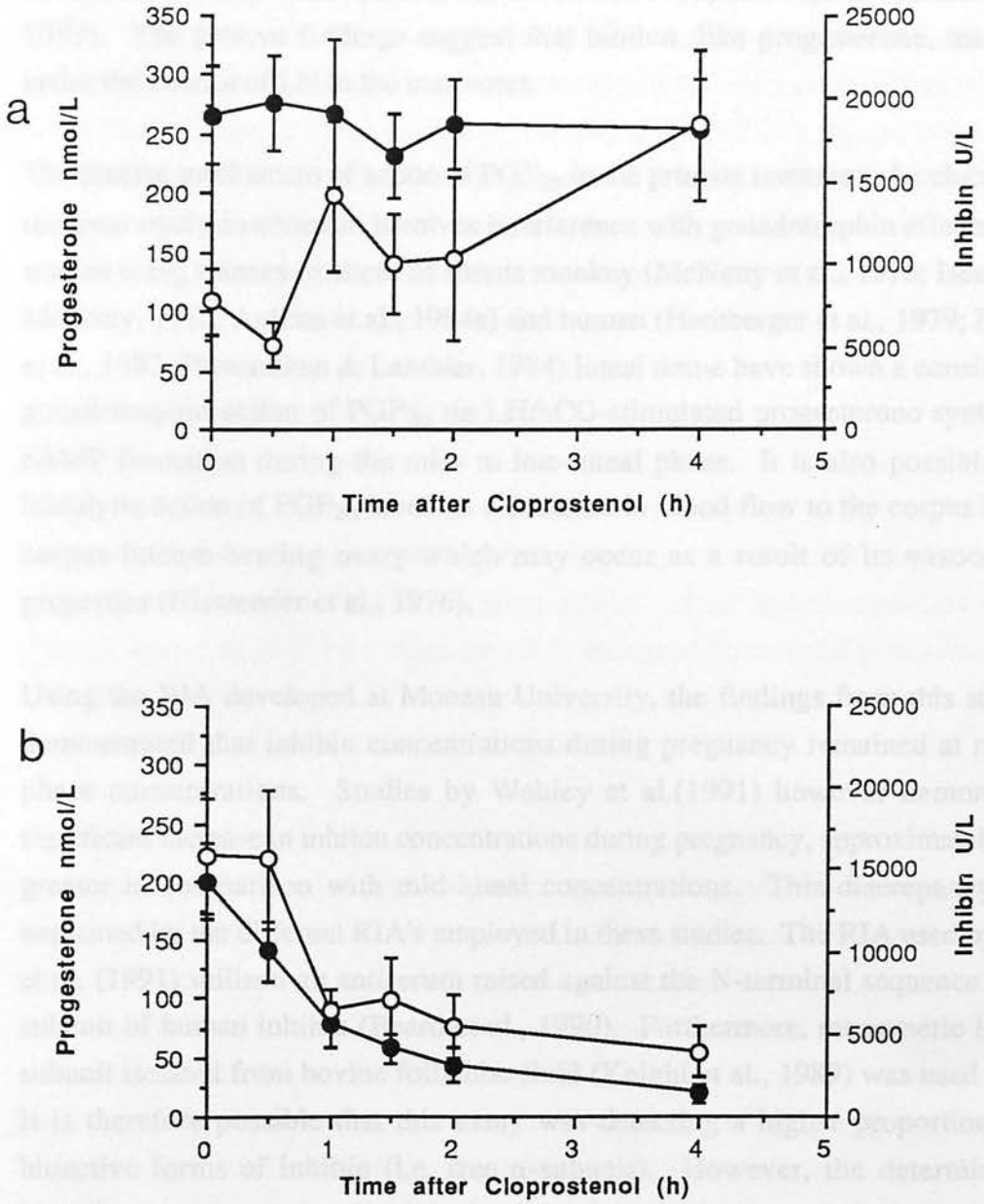


Fig. 2.17. Progesterone (●) and inhibin (O) concentrations in control marmosets (a) (n=3) and marmosets treated with cloprostenol (b) (n=4) on days 17-20 after ovulation. Values are means ± S.E.M.

demonstrated that progesterone is under the control of LH in this species , similar to the situation in Old World primates (Fraser et al., 1989) and women (McLachlan et al., 1989). The present findings suggest that inhibin, like progesterone, may also be under the control of LH in the marmoset.

The precise mechanism of action of $PGF_{2\alpha}$ in the primate remains to be elucidated but the most likely mechanism involves interference with gonadotrophin effects. Several studies using minces or slices of rhesus monkey (McNatty et al., 1975; Henderson & McNatty, 1977; Auletta et al., 1984a) and human (Hamberger et al., 1979; Dennefors et al., 1982; Patwardhan & Lanthier, 1984) luteal tissue have shown a consistent anti-gonadotrophin action of $PGF_{2\alpha}$ on LH/hCG-stimulated progesterone synthesis and cAMP formation during the mid- to late-luteal phase. It is also possible that the luteolytic action of $PGF_{2\alpha}$ involves a decrease in blood flow to the corpus luteum or corpus luteum-bearing ovary which may occur as a result of its vasoconstrictor properties (Niswender et al., 1976).

Using the RIA developed at Monash University, the findings from this study have demonstrated that inhibin concentrations during pregnancy remained at mid-luteal phase concentrations. Studies by Webley et al.(1991) however demonstrated a significant increase in inhibin concentrations during pregnancy, approximately 1.8 fold greater in comparison with mid-luteal concentrations. This discrepancy may be explained by the different RIA's employed in these studies. The RIA used by Webley et al. (1991) utilized an antiserum raised against the N-terminal sequence of the α-subunit of human inhibin (Beard et al., 1990). Furthermore, monomeric inhibin α-subunit isolated from bovine follicular fluid (Knight et al., 1989) was used as tracer. It is therefore possible that this assay was detecting a higher proportion of non-bioactive forms of inhibin (i.e. free α-subunit). However, the determination of bioactive concentrations of inhibin throughout the ovulatory cycle in the marmoset are necessary to confirm this.

Part III- Inhibin secretion in the human.

2.3.1 Clinical Study 1. The source of inhibin secretion during the human menstrual cycle.

To complement the *in vivo* experiments using the stumptailed macaque and common marmoset monkey described in Parts I and II, I collaborated in two clinical studies in women to investigate inhibin secretion.

The aim of this study was to determine the source of inhibin secretion during the normal human menstrual cycle. As with other hormones, conclusive evidence for the source of secretion would be the demonstration of either an increased concentration in the venous effluent of the proposed site of release or a fall in peripheral concentration after its removal. In this study we have therefore measured the inhibin concentrations in the peripheral and ovarian veins of women undergoing laparotomy at different stages of the menstrual cycle as well as measuring the peripheral inhibin concentration before and for 24h after enucleation of the corpus luteum.

2.3.2 Materials and Methods

(i) Subjects

Forty-one women aged between 29 and 45 (median 39.4) years undergoing hysterectomy were studied. All subjects were in good health, had regular menstrual cycles (26-35 days) and had two healthy ovaries. None had received any hormonal therapy within the previous three months. Informed consent was obtained from all subjects and ethical approval for the study was obtained from the Lothian Area Ethical Committee, Reproductive Medicine Subcommittee, Edinburgh.

(ii) Cycle dating

Where possible (17 out of the 24 luteal phase subjects), subjects were recruited prior to admission and daily urine samples were collected by these subjects throughout the cycle of ovarian vein sampling. The LH concentration was subsequently assayed in these samples by Dr.Peter Illingworth and the day of ovulation taken as the day of the urinary LH peak. In addition, the stage of the cycle was also determined from the histological appearance of endometrial sections obtained during surgery (Noyes et al., 1950; Hertig & Rock, 1950; Li et al., 1988) and the date of the last menstrual period.

The stage of the menstrual cycle for the subjects in the follicular phase was classed as early follicular (EF) when they were within 7 days of their last menstrual period with no follicle present greater than 10mm diameter (n=7) and as late follicular (LF) when there was a follicle present of more than 10mm diameter (n=7).The subjects in the luteal phase were grouped according to the time since ovulation as follows: early luteal (EL), 1-4 days since ovulation (n=9); mid luteal (ML), 5-9 days since ovulation (n=9); late luteal (LL), 10-14 days since ovulation (n=9).

(iii) Collection of ovarian vein samples

In order to examine the effects of general anaesthesia on inhibin levels, a sample was collected prior to anaesthesia in a group of 13 subjects at different stages of the menstrual cycle. Samples were then collected simultaneously at the time of operation from a peripheral vein and the veins draining both ovaries. Ovarian vein samples were obtained according to the method described by Baird & Fraser (1975) by Dr. Peter Illingworth. The samples were collected into heparinised tubes, separated by centrifugation for 15 min at 800g and stored at -20°C until assayed for oestradiol, progesterone and inhibin. The peripheral samples were in addition assayed for gonadotrophins by Dr.Peter Illingworth.

(iv) Measurement of hormone concentrations after enucleation of the corpus luteum.

In 13 of the subjects (4 EL, 5 ML, 4 LL), the corpus luteum was enucleated at the time of operation after collection of the ovarian vein samples. Peripheral samples were then obtained through an indwelling cannula at 30, 60, 120, 240, 480 and 1440 min after luteectomy. These samples were subsequently processed as above and assayed for inhibin, progesterone, LH and FSH.

(v) Assays

Inhibin, progesterone and oestradiol were measured by radioimmunoassay described previously in Part I of this chapter and in chapter 4. LH and FSH were measured by Dr.Peter Illingworth using radioimmunoassays previously described (Backstrom et al., 1982).

(vi) Statistical analyses

In the calculation of results a log-normal distribution was assumed. Results are expressed as geometric means with 67% confidence intervals and all subsequent statistical analysis was carried out on logarithmically transformed data. The ovarian data were assessed by two-way analysis of variance (ANOVA) using a commercial statistics software package (CLR Anova, Clear Lake Research) to examine the effects of cycle stage (between-subject variable) and site of sampling (within-subject variable) on hormone concentrations. Where the initial analysis revealed a statistically significant effect, this effect was further investigated with Duncan's multiple range tests. The data on peripheral concentrations after luteectomy were examined by one-way ANOVA using the time of collection as a within-subject variable. Where this

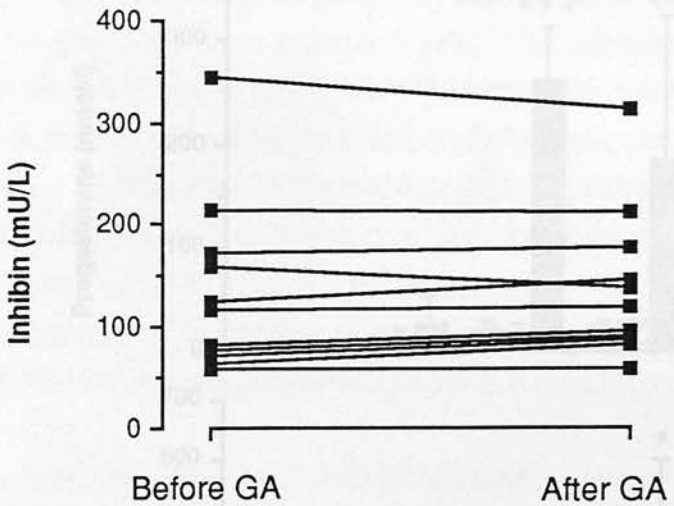


Fig. 2.18. Individual peripheral inhibin concentrations in 13 subjects before and during general anaesthesia.

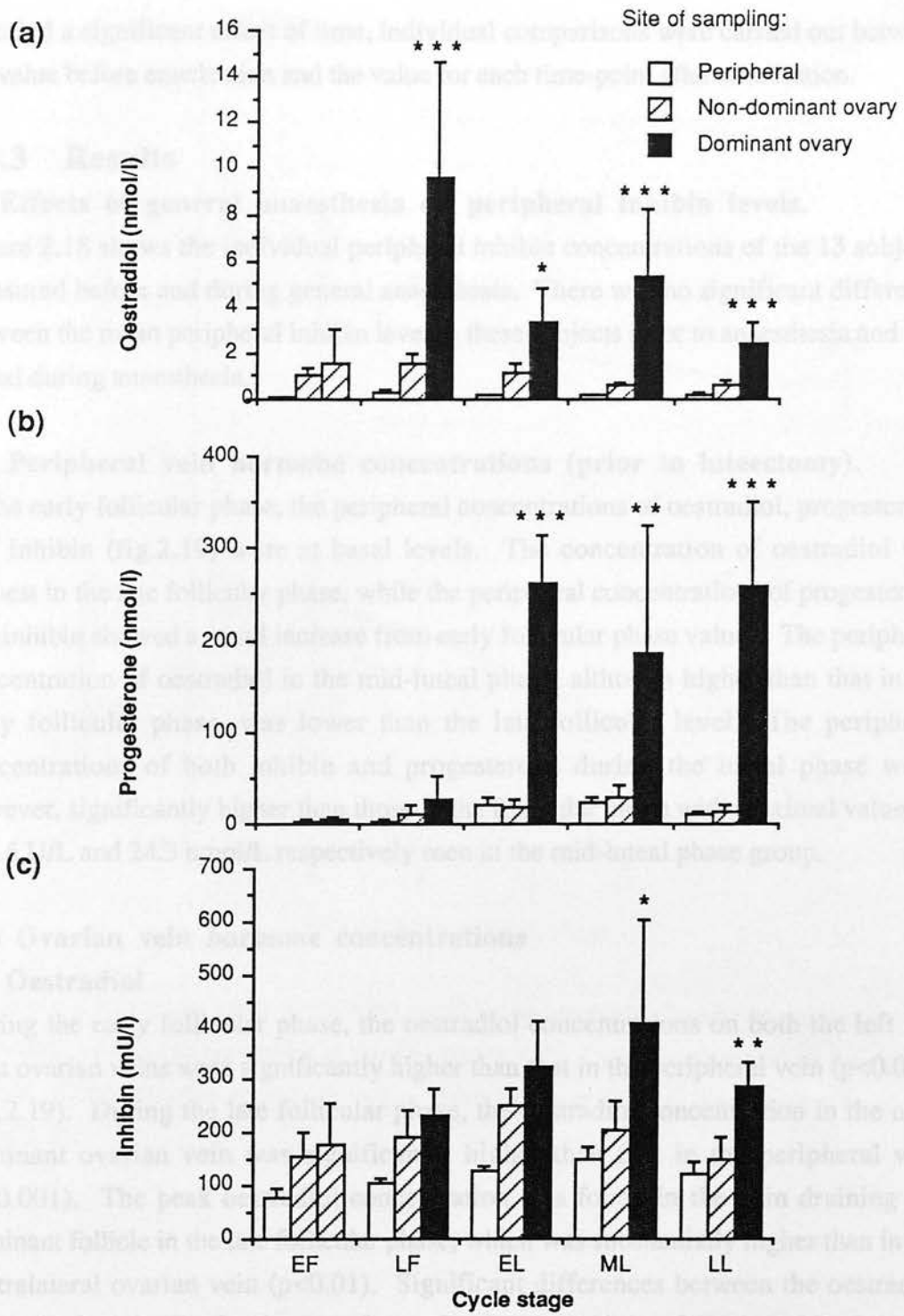


Fig. 2.19. Geometric mean (with 67% confidence limits shown) concentrations of (a) oestradiol, (b) progesterone and (c) inhibin in the peripheral, non-dominant and dominant ovarian veins during the early follicular (EF), late follicular (LF), early luteal (EL), mid-luteal (ML) and late luteal (LL) stages of the menstrual cycle. In the early follicular phase, where dominance is not yet apparent, the data shown refer to the right and left ovaries. Non-dominant ovarian vein v. dominant ovarian vein: * $p<0.02$, ** $p<0.01$, *** $p<0.001$.

revealed a significant effect of time, individual comparisons were carried out between the value before enucleation and the value for each time-point after enucleation.

2.3.3 Results

(i) Effects of general anaesthesia on peripheral inhibin levels.

Figure 2.18 shows the individual peripheral inhibin concentrations of the 13 subjects measured before and during general anaesthesia. There was no significant difference between the mean peripheral inhibin level in these subjects prior to anaesthesia and that found during anaesthesia.

(ii) Peripheral vein hormone concentrations (prior to luteectomy).

In the early follicular phase, the peripheral concentrations of oestradiol, progesterone and inhibin (fig.2.19) were at basal levels. The concentration of oestradiol was highest in the late follicular phase, while the peripheral concentrations of progesterone and inhibin showed a small increase from early follicular phase values. The peripheral concentration of oestradiol in the mid-luteal phase, although higher than that in the early follicular phase, was lower than the late follicular level. The peripheral concentrations of both inhibin and progesterone during the luteal phase were, however, significantly higher than those in the follicular phase with maximal values of 152.5 U/L and 24.3 nmol/L respectively seen in the mid-luteal phase group.

(iii) Ovarian vein hormone concentrations

(a) Oestradiol

During the early follicular phase, the oestradiol concentrations on both the left and right ovarian veins were significantly higher than that in the peripheral vein ($p<0.001$) (fig.2.19). During the late follicular phase, the oestradiol concentration in the non-dominant ovarian vein was significantly higher than that in the peripheral vein ($p<0.001$). The peak oestradiol concentration was found in the vein draining the dominant follicle in the late follicular phase, which was substantially higher than in the contralateral ovarian vein ($p<0.01$). Significant differences between the oestradiol concentrations in the dominant and non-dominant ovarian veins were also apparent during the early-($p<0.02$), mid-($p<0.001$) and late-($p<0.001$) luteal phases.

(b) Progesterone

No differences were observed between the progesterone concentrations in the non-dominant ovarian veins and peripheral veins at any stage of the cycle (fig.2.19). There was similarly no significant difference between the progesterone concentrations in the

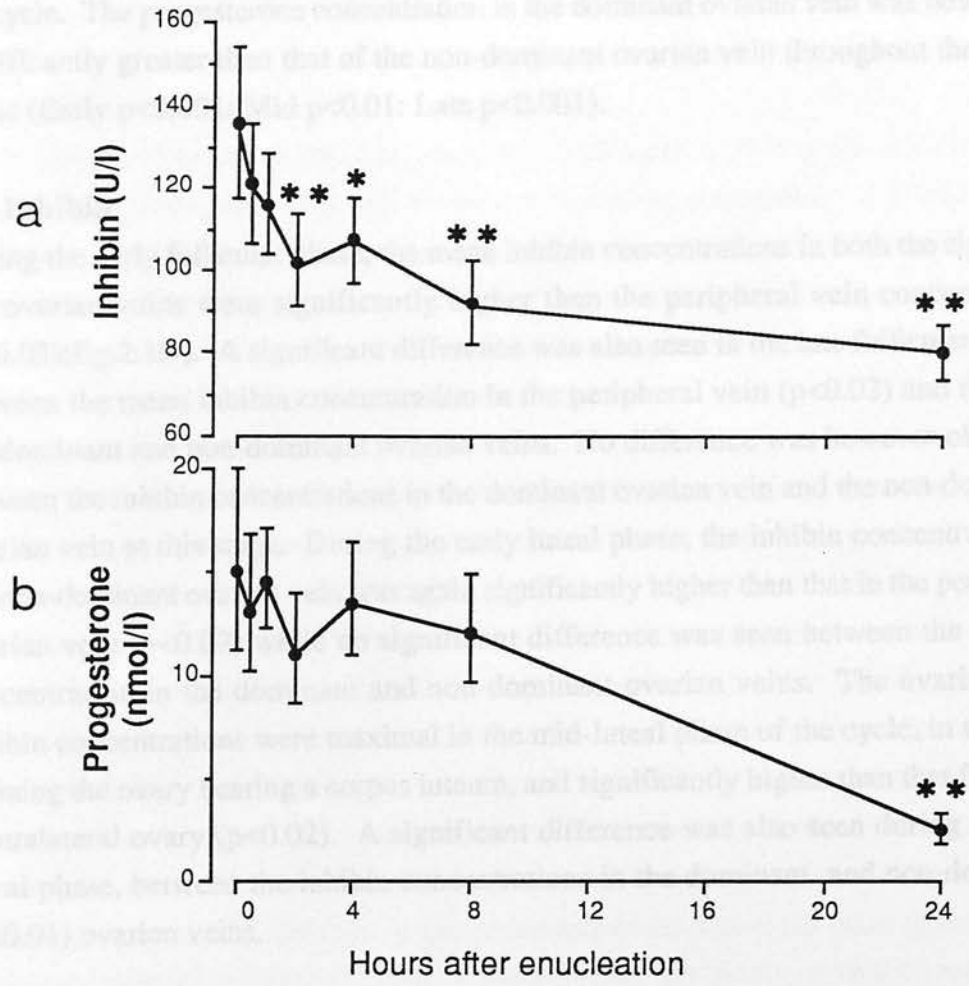


Fig. 2.20. Geometric mean (with 67% confidence intervals shown) concentrations of (a) inhibin and (b) progesterone at time intervals shown after enucleation of the corpus luteum. Concentration at time shown v. concentration prior to luteectomy: *$p<0.05$, ** $p<0.01$.

dominant and non-dominant ovarian veins during the early or late follicular phases of the cycle. The progesterone concentration in the dominant ovarian vein was however significantly greater than that of the non-dominant ovarian vein throughout the luteal phase (Early $p<0.001$: Mid $p<0.01$: Late $p<0.001$).

(c) Inhibin

During the early follicular phase, the mean inhibin concentrations in both the right and left ovarian veins were significantly higher than the peripheral vein concentration ($p<0.02$)(fig.2.19). A significant difference was also seen in the late follicular phase, between the mean inhibin concentration in the peripheral vein ($p<0.02$) and those in the dominant and non-dominant ovarian veins. No difference was however observed between the inhibin concentrations in the dominant ovarian vein and the non-dominant ovarian vein at this stage. During the early luteal phase, the inhibin concentration in the non-dominant ovarian vein was again significantly higher than that in the peripheral ovarian vein ($p<0.02$) while no significant difference was seen between the inhibin concentration in the dominant and non-dominant ovarian veins. The ovarian vein inhibin concentrations were maximal in the mid-luteal phase of the cycle, in the vein draining the ovary bearing a corpus luteum, and significantly higher than that from the contralateral ovary ($p<0.02$). A significant difference was also seen during the late luteal phase, between the inhibin concentrations in the dominant and non-dominant ($p<0.01$) ovarian veins.

(iv) Peripheral concentrations following enucleation of the corpus luteum

Following enucleation of the corpus luteum, there was a fall in the peripheral concentration of inhibin (fig.2.20) from a mean value in these women of 134.4 U/L prior to luteectomy to a level of 80.0 U/L at 24 hours after operation. There was a highly significant effect of time after enucleation on the inhibin concentration ($p<0.001$) with the inhibin concentration showing a significant change from time 0 at 120 ($p<0.01$), 240 ($p<0.05$), 480 and 1440 min ($p<0.01$). An approximate estimate of the half-life of the initial fall was obtained by taking the inhibin concentrations at 24h as representative of the basal inhibin concentrations after luteectomy. The half-life of the initial component of the disappearance curve was obtained from the slope of the resulting logarithmically transformed values. When calculated in this way, the first component of the half-life of inhibin was found to be 86.1 min (S.E.10.8min). There was also a significant effect of time on the peripheral progesterone concentration

($p<0.001$) with the level falling from 14.97 nmol/L prior to operation to 2.43 nmol/L at 24 h.

2.3.4 Discussion

The observations in this study of higher concentrations of inhibin in the veins draining the corpus luteum, and of a fall in the peripheral concentration of inhibin after luteectomy, provides conclusive evidence for the secretion of inhibin into the circulation by the human corpus luteum. These findings are in agreement with previous data (McLachlan et al., 1987a) demonstrating that the peak values for inhibin in peripheral blood are seen in the luteal phase of women.

Although there was significantly more inhibin in the ovarian veins than in the peripheral veins, the apparent magnitude of the difference was not as great as that seen for the sex steroids. This probably reflects differences between the relative half-lives of the respective hormones. While the metabolic clearance rates for oestradiol and progesterone are 1350 L/day and 2200 L/day respectively, that for inhibin appears to be substantially slower with a value as low as 21.3 ml/min having been reported in the sheep (McNeilly et al., 1989). The half-life of the initial decline in peripheral inhibin concentration after luteectomy in this study was found to be 86min. This calculation was based on the assumption that the fall in inhibin concentration follows a single exponential equation. However, it has previously been shown for other glycoprotein hormones that the plasma disappearance curve is the result of two exponentials comprising an initial rapid component and a second slower component (Yen et al., 1968). This may also be the case for inhibin, but due to the limited number of time points in this experiment, it was only possible to perform a limited calculation to obtain a crude estimate of the initial rapid component of inhibin clearance.

The difference in the early follicular phase between the inhibin concentrations in the ovarian and peripheral veins demonstrates that the ovary secretes inhibin at this time even though the only follicles present are small and immature. It was also found however, that while the dominant follicle of the late follicular phase secretes massive amounts of oestradiol, there was no evidence of a significant increase in inhibin secretion by the dominant follicle. This finding was unexpected since follicular fluid contains very large amounts of inhibin. In addition, it has previously been shown in women that follicular fluid inhibin bioactivity is related to the degree of maturation of the follicle (Marrs et al., 1984) and in marmosets that granulosa cells from larger

follicles show increased inhibin secretion *in vitro* both basally and in response to FSH (Hillier et al., 1989).

Considered along with the *in vivo* observation that the peripheral concentration of inhibin remains unchanged during the early follicular phase, the absence of significant inhibin secretion by the dominant follicle is further evidence that inhibin is not the factor responsible for the fall in plasma FSH concentration seen during the follicular phase of the cycle.

2.3.5 Clinical Study 2. Inhibin production by the corpus luteum following 'pharmacological rescue' by hCG.

The aim of the second clinical study was to further investigate the secretion of inhibin in the human by measuring the concentration of inhibin following 'pharmacological rescue' of the corpus luteum with increasing doses of exogenous hCG.

2.3.6 Materials and Methods

(i) Subjects

Four healthy volunteers aged from 25-33 years were recruited for the study. All had a history of regular menstrual cycles of 25-35 days, were within 10% of ideal body weight and none were taking any form of drug therapy or had taken any form of steroidal contraception within the previous three months. Informed consent was obtained from all subjects and ethical approval was obtained from the Lothian Area Ethical Committee, Reproductive Medicine Subcommittee, Edinburgh.

(ii) Protocol

Daily blood samples (10ml) were collected from each subject for one control menstrual cycle prior to treatment with hCG. The timing of the mid-cycle gonadotrophin surge was monitored prospectively by daily rapid LH RIA (Djahanbakhch et al., 1981). Daily blood samples were continued into a second cycle and hCG (Profasi, Serono) was administered by intramuscular injection starting 7 days after the LH surge. hCG was administered for seven consecutive days in incremental doses of 125, 250, 500, 1000, 2000, 4000 and 8000 IU and blood sampling continued for the remainder of the cycle until the next LH surge. These procedures were performed by Dr.Peter Illingworth. The plasma was separated by centrifugation and stored at -20°C.

(iii) Assays

The plasma samples were assayed for progesterone, oestradiol and inhibin using methods previously described in this chapter. Some of the inhibin samples were assayed by Dr.Kogie Reddi. The samples were measured for LH, FSH and hCG by immunoradiometric assays using commercially obtained kits (Maiaclone, Serono UK Ltd.) by Dr.Peter Illingworth.

(iv) Statistical analyses

A log-normal distribution was assumed in the calculation of results. They are presented as geometric means with 67% confidence intervals and all subsequent statistical analyses were carried out on logarithmically transformed data. The effect of hCG on circulating hormone levels was evaluated by two-way analysis of variance using a commercial statistics software package (CLR Anova, Clear Lake Research).

In this analysis, days 1-9 (before the rise in plasma hCG levels) and days 10-19 (after the rise in plasma hCG levels) were considered separately and the day and hCG treatment assigned as within-subject variables. Where this initial analysis demonstrated a significant effect of hCG treatment, individual results for each day were compared by paired t-test.

2.3.7 Results

(i) Control cycle

Figure 2.21 shows the mean plasma concentrations of inhibin, progesterone and oestradiol in all four subjects during the control cycle. The results are expressed relative to the previous and following LH surges. The inhibin concentrations increased to a plateau between days 5 and 9 after the LH surge, reaching a maximum level of 159 U/l on day 9. Inhibin concentrations then returned to early follicular phase levels by day 14 after the LH peak. There was no change in peripheral concentrations of inhibin during the follicular phase of the cycle until two days prior to the LH peak, when concentrations began to rise. In the follicular phase there was a progressive increase in the concentration of oestradiol. There was a rise in both progesterone and oestradiol towards the mid-luteal phase with a fall to early follicular phase levels by 14 days after the LH peak.

(ii) hCG treated cycle

Figure 2.22 shows the mean plasma concentrations of inhibin, progesterone and oestradiol in hCG-treated cycles from subjects 1-3. The equivalent concentration for

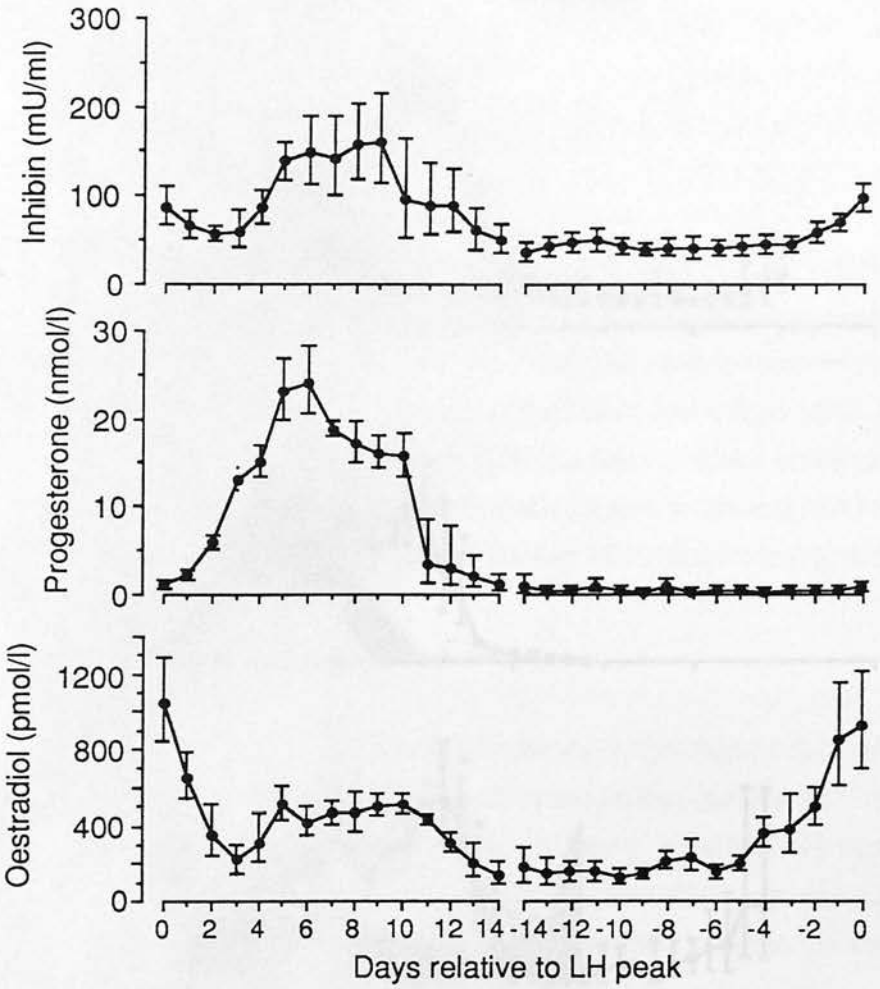


Fig. 2.21. Geometric mean (with 67% confidence intervals) plasma concentrations of inhibin, progesterone and oestradiol for the four subjects during the control cycle. Data from days 0 to 14 are normalized around the preceding midcycle LH peak whilst those from days -14 to 0 are normalized around the succeeding midcycle LH peak.

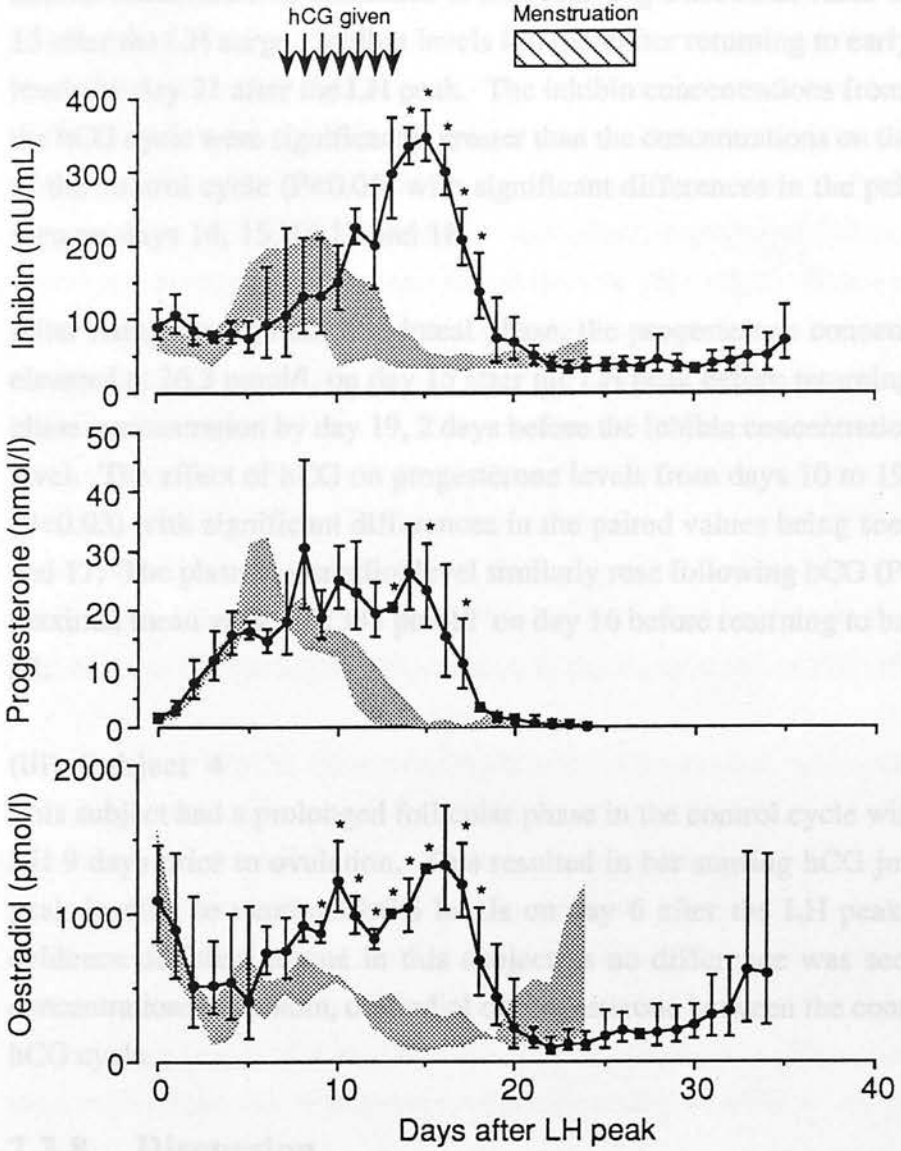


Fig. 2.22. Geometric mean (67% confidence intervals) plasma concentrations of inhibin, progesterone and oestradiol in the three subjects who received hCG commencing day 7 after the midcycle LH peak. Data are normalized around the preceding midcycle LH peak. The shaded areas represent the upper and lower 67% confidence intervals for geometric mean values of the same subjects during the control cycle. The arrows signify the days on which the incremental doses of hCG were being administered and the hatched area is the time of menstruation in the hCG cycle. (hCG v. control cycle, *$p<0.05$)

the same day in the control cycle is shown in the shaded area. As in the control cycle, inhibin concentrations rose in the mid-luteal phase. Following hCG treatment, the inhibin concentrations continued to rise, reaching a maximal value of 347 U/l on day 15 after the LH surge. Inhibin levels fell thereafter returning to early follicular phase levels by day 21 after the LH peak. The inhibin concentrations from days 10 to 19 in the hCG cycle were significantly greater than the concentrations on the equivalent days of the control cycle ($P<0.05$) with significant differences in the paired values being seen on days 14, 15, 16,17 and 18.

After rising through the mid-luteal phase, the progesterone concentration remained elevated at 26.3 nmol/L on day 15 after the LH peak before returning to the follicular phase concentration by day 19, 2 days before the inhibin concentration returned to this level. The effect of hCG on progesterone levels from days 10 to 19 was significant, ($P<0.05$) with significant differences in the paired values being seen on days 13, 15 and 17. The plasma oestradiol level similarly rose following hCG ($P<0.05$) to reach a maximal mean value of 1333 pmol/l on day 16 before returning to basal levels by day 22.

(iii) Subject 4

This subject had a prolonged follicular phase in the control cycle with a false peak of LH 9 days prior to ovulation. This resulted in her starting hCG just before the LH peak leading to maximal hCG levels on day 6 after the LH peak. There was no evidence of luteal rescue in this subject as no difference was seen in the plasma concentrations of inhibin, oestradiol or progesterone between the control cycle and the hCG cycle.

2.3.8 Discussion

The concentrations of inhibin found during the control menstrual cycle were similar to those reported previously (McLachlan et al., 1987a; Robertson et al., 1988a). The concentrations reported in this study are however substantially lower in absolute terms in comparison with these previous studies. The explanation for this discrepancy is that our in-house partially purified human follicular fluid standard is of a different calibrated bioactivity (Reddi et al., 1989). This finding emphasizes the requirement for a universal reference standard preparation.

The findings from this study demonstrate that the human corpus luteum responds to stimulation with hCG by producing significantly increased amounts of inhibin. This

confirms that the corpus luteum is an important source of inhibin in early pregnancy. There are two previous reports of plasma concentrations of inhibin in early pregnancy (McLachlan et al., 1987b,c). In the first study, the luteal response may be atypical, as it was carried out on women who had undergone a superovulation induction for the purposes of *in-vitro* fertilization followed by follicular aspiration. In the second study, raised inhibin levels were found in agonadal women who had conceived by means of *in-vitro* fertilization with steroidal support. It was thus concluded that inhibin in early pregnancy largely originates from the trophoblast, implying that the corpus luteum is a relatively unimportant source of inhibin at this time. However, most of the measurements of inhibin concentrations in the agonadal women were carried out in later pregnancy and there was only one measurement before 28 days following embryo transfer. It is possible that inhibin secretion from the corpus luteum is a short-lived phenomenon in the very early days of pregnancy before being superseded by hormone of trophoblastic origin. The maximal plasma concentrations of inhibin during the luteal phase are however coincident with suppression of the plasma FSH level at this time, and the further suppression of FSH levels following luteal rescue highlights the role of luteal products, possibly inhibin, in the suppression of FSH after conception.

Tsonis et al (1987b) demonstrated that *in vitro* release of inhibin by luteinized granulosa cells is stimulated by LH but not FSH. This study has demonstrated that injection of hCG resulted in a marked increase in the plasma concentration of inhibin, providing further evidence that inhibin release from the corpus luteum is controlled through the LH receptor. Although the intracellular mechanism mediating this response remains uncertain, the involvement of cAMP is likely as it is known that inhibin release from follicular granulosa cells is cAMP dependent (Bicsak et al., 1986) as are the parallel responses of progesterone and oestradiol to hCG.

Chapter 3

Control of Inhibin production *in vivo* and *in vitro*

The aims of the studies in this chapter were firstly to examine the control of progesterone and inhibin secretion *in vivo* in the stumptailed macaque using an LHRH antagonist with and without replacement of gonadotrophin and secondly, to investigate the control of inhibin secretion *in vitro* using a cell culture system which would maintain the growth of steroidogenically active human luteal cells.

Part 1. Control of progesterone and inhibin secretion during the luteal phase in the macaque

3.1.1 Introduction

A convenient approach for investigations of the gonadotrophic control of luteal function is by treatment with luteinizing hormone-releasing hormone (LHRH) antagonists to block pituitary secretion of LH and FSH. Various studies involving the use of LHRH antagonists have established that progesterone secretion by the corpus luteum of women (Mais et al., 1986), Old World primates (Collins et al., 1986; Fraser et al., 1985; 1986; 1987a) and New World primates (Hodges et al., 1988) is dependent on pituitary LH secretion . Treatment with a LHRH antagonist for 3 days, starting during the mid luteal phase, also caused permanent suppression of serum inhibin concentrations for the remainder of the cycle, as demonstrated in chapter 2 and by Fraser et al. (1989) and McLachlan et al. (1989). The ability of the corpus luteum to recover from transitory suppression of gonadotrophin secretion has also been investigated in monkeys with induced hypothalamic lesions. In the rhesus monkey, where endogenous gonadotrophin was abolished using radiofrequency lesions and restored by chronic pulsatile infusion of LHRH, withdrawal of gonadotrophic support for 3 days resulted in luteolysis or recovery of luteal function depending on the age of the corpus luteum (Hutchison & Zeleznik, 1985).

The aim of the following *in vivo* studies in the stumptailed macaque were threefold. Firstly, to examine the temporal relationship between the serum concentrations of inhibin and progesterone after LHRH antagonist treatment; secondly, since treatment with LHRH antagonist for 3 days during the mid-luteal phase to induce continued luteal suppression can be used to test the luteal response to exogenous factors, it was

determined whether suppression of inhibin secretion induced by LHRH antagonist treatment in the macaque could be prevented with either human chorionic gonadotrophin (hCG) or FSH and finally, since the results of Hutchison & Zeleznik (1985) indicated that transitory suppression of LHRH during the mid-luteal phase could create a period of suppression of luteal function followed by recovery to normal for the remainder of the luteal phase, it was investigated whether this could be achieved by reducing the administration of LHRH antagonist to 1 or 2 days. Induction of such a response could be used to identify the action of putative luteolytic agents, the effects of which might be obscured in the presence of endogenous gonadotrophins, but which could have a deleterious effect on luteal function when gonadotrophins were transitorily suppressed, resulting in abolition of the recovery phase. Such an approach might help to identify strategies for overcoming the ability of hCG to "rescue" the corpus luteum and lead to improved methods of post-ovulatory fertility control.

3.1.2 Materials and Methods

3.1.2.1 Animals

Eighteen adult female macaques (*Macaca arctoides*) weighing 8-13kg were used in these studies. All animals demonstrated regular menstrual cycles with normal luteal phases as determined by hormonal estimations three times per week, fulfilling the criteria previously described in chapter 2. When animals were used in more than one aspect of the study, at least 3 months were allowed to elapse between treatment intervals. Blood samples (4ml) were collected daily throughout the late follicular phase and luteal phase as described in chapter 2. The day of the LH surge was considered as day 0 of the luteal phase if followed immediately by an increase in subsequent daily serum progesterone concentrations.

3.1.2.2 Treatment with an LHRH antagonist.

To determine the ability of the corpus luteum to recover from various periods of suppression of gonadotrophin, macaques were injected with the LHRH antagonist [N-Ac-D-Nal(2)^1,D-pCl-Phe^2,D-Trp^3,D-hArg(Et$_2$)^6,D-Ala^{10}]LHRH (Detirelix:Syntex, Palo Alto, CA, USA.) dissolved in 0.9% NaCl/propylene glycol (1:1v/v) and administered s.c. at a dose of 300µg/kg, once daily for 1, 2 or 3 days (n=6 per group), beginning on day 6-8 after the mid-cycle LH surge. Previous studies have shown that 300µg/kg causes suppression of the pituitary-gonadal axis for 24h in both female and male monkeys, while lower doses result in partial recovery of gonadal

steroid production during the 24h period (Fraser et al., 1985; 1986; Adams et al., 1986). Daily samples were collected throughout the luteal phase with samples collected at 0,2,4,6,8 and 12h from the time of first administration of antagonist in 12 of the treated animals. Six macaques receiving vehicle alone and studied over the same time period acted as controls. Blood samples were centrifuged at 1000g for 30 min and stored at -20°C until assayed for progesterone, LH and inhibin. Luteal function was considered to have recovered if serum progesterone concentrations rose to >5nmol/L for 2 consecutive days after starting treatment.

3.1.2.3 Gonadotrophin replacement

To elucidate the gonadotrophic control of the corpus luteum further, six macaques were treated with 300μg antagonist /kg once daily for 3 consecutive days beginning on day 6-8 of the luteal phase i.e. a regimen shown previously to produce a permanent suppression of luteal progesterone and inhibin secretion (chapter 2). In addition, they received concomitantly either hCG (Chorulon, Intervet, Cambridge) in incremental doses of 30,60,90,180 and 360 IU i.m. for 5 days, or FSH (Metrodin, Serono Laboratories (U.K.) Ltd., Welwyn Garden City, Herts.) at 36 IU/ day for 5 days (n=3 per group). Blood samples were collected daily throughout the luteal phase.

3.1.2.4 Assays

Occurrence of ovulation was determined by use of a rapid progesterone radioimmunoassay, together with an LH radioimmunoassay, to determine the day of the mid-cycle LH surge as described in chapter 2. Inhibin concentrations were measured using a heterologous radioimmunoassay as described in chapter 2.

3.1.2.5 Statistical analyses

Data were subjected to one and two factor analyses of variance (ANOVA) following log transformation to reduce heterogeneity of variance. Where significant differences were observed, data were further analysed using Newman-Keuls test.

3.1.3 Results

The short term (24h) response of progesterone and inhibin to the effects of LHRH antagonist treatment are shown in figure 3.1. Serum progesterone was significantly ($p<0.01$) suppressed by 6 h after injection while inhibin was not significantly affected during the 24h period. Thereafter, progesterone concentrations remained significantly

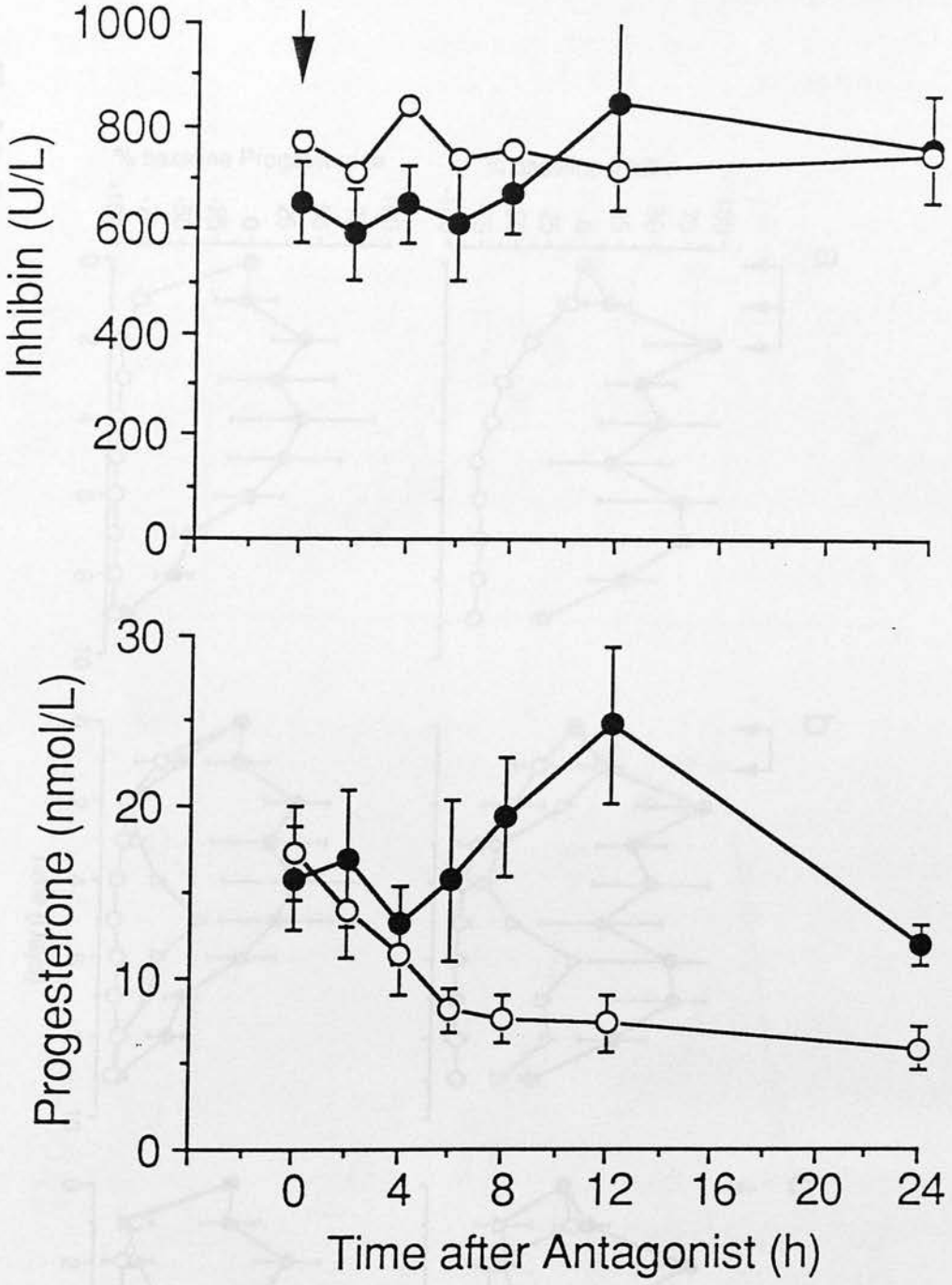


Fig. 3.1. Serum concentrations of progesterone and inhibin during the mid-luteal phase in control macaques (●) (n=6) and in macaques receiving 300μg/kg LHRH antagonist (arrow) (O) (n=12). Values are means ± S.E.M.

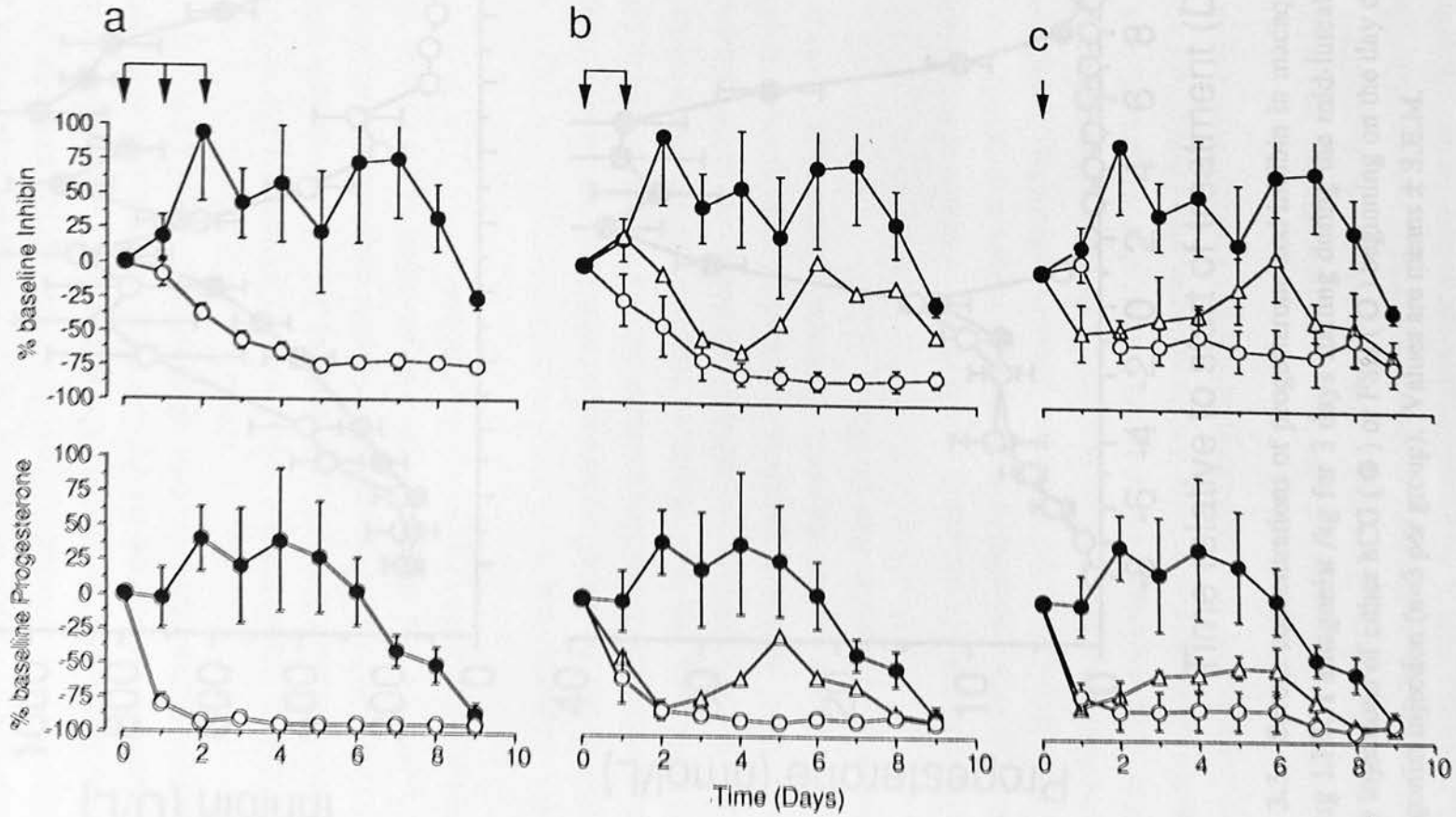


Fig. 3.2. Serum concentrations of progesterone and inhibin, expressed as % baseline levels in control macaques (●), and in animals receiving s.c. injections of 300μg LHRH antagonist /kg during the mid-luteal phase for (a) 3, (b) 2 or (c) 1 day (arrows). Each group consisted of six animals. In the animals treated for 1 or 2 days, data are divided according to whether progesterone secretion remained suppressed (O) or demonstrated recovery (△). Values are means ± S.E.M.

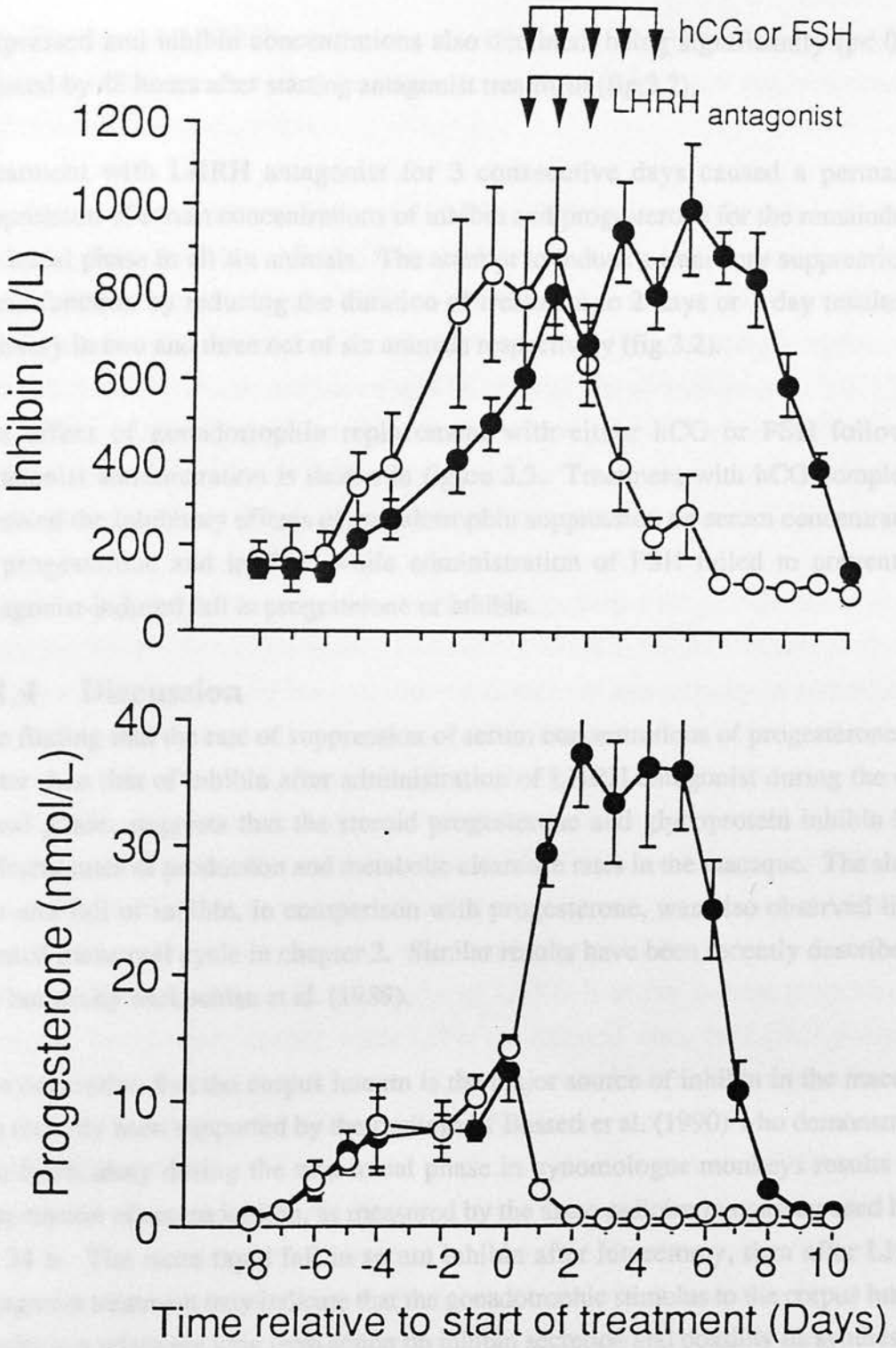


Fig. 3.3. Serum concentrations of progesterone and inhibin in macaques receiving 300µg LHRH antagonist /kg for 3 days starting during the mid-luteal phase and 5 daily injections of either hCG (●) or FSH (O) beginning on the day of first LHRH antagonist injection (n=3 per group). Values are means ± S.E.M.

suppressed and inhibin concentrations also declined, being significantly ($p< 0.01$) reduced by 48 hours after starting antagonist treatment (fig.3.2).

Treatment with LHRH antagonist for 3 consecutive days caused a permanent suppression of serum concentrations of inhibin and progesterone for the remainder of the luteal phase in all six animals. The attempt to induce a transitory suppression of luteal function by reducing the duration of treatment to 2 days or 1 day resulted in recovery in two and three out of six animals respectively (fig.3.2).

The effect of gonadotrophin replacement with either hCG or FSH following antagonist administration is shown in figure 3.3. Treatment with hCG completely reversed the inhibitory effects of gonadotrophin suppression on serum concentrations of progesterone and inhibin, while administration of FSH failed to prevent the antagonist-induced fall in progesterone or inhibin.

3.1.4 Discussion

The finding that the rate of suppression of serum concentrations of progesterone was faster than that of inhibin after administration of LHRH antagonist during the mid-luteal phase, suggests that the steroid progesterone and glycoprotein inhibin have different rates of production and metabolic clearance rates in the macaque. The slower rise and fall of inhibin, in comparison with progesterone, was also observed in the normal menstrual cycle in chapter 2. Similar results have been recently described in the human by McLachlan et al. (1989).

The contention that the corpus luteum is the major source of inhibin in the macaque has recently been supported by the findings of Basseti et al. (1990) who demonstrated that luteectomy during the mid-luteal phase in cynomologus monkeys results in a suppression of serum inhibin, as measured by the same radioimmunoassay used here, by 24 h. The more rapid fall in serum inhibin after luteectomy, than after LHRH antagonist treatment may indicate that the gonadotrophic stimulus to the corpus luteum results in a relatively long term action on inhibin secretion and possibly its synthesis.

The complete and sustained suppression of progesterone and inhibin secretion obtained by gonadotrophin withdrawal by three daily injections of LHRH antagonist was overcome by concomitant administration of hCG but not FSH. The conclusion that LH but not FSH is the gonadotrophin controlling inhibin secretion during the luteal phase in primates is supported by *in vitro* studies on human luteinized granulosa

cells (Tsonis et al., 1987b) and a recent study in which an LHRH antagonist was administered to women during the luteal phase of the cycle with a single injection of hCG (McLachlan et al., 1989).

By reducing the duration of treatment to 1 or 2 days, we investigated whether a recovery to normal luteal function would occur. A corpus luteum deprived of gonadotrophin support by an LHRH antagonist could provide a "window" within the luteal phase during which the action of putative luteotrophic or luteolytic agents could be compared. For a valid model it would be necessary to administer sufficient LHRH antagonist to achieve a marked suppression of luteal function over a period of 1-2 days. Administration of 300μg/kg antagonist for 2 days was successful in suppressing serum concentrations of progesterone and inhibin to follicular phase levels during this period. However, in only two of six animals did serum progesterone return to the normal luteal phase range during the post-treatment period. Reducing the duration of antagonist treatment resulted in partial recovery in three of six macaques. A recovery has been observed in the rhesus monkey in which LHRH stimulation was controlled by exogenous LHRH which was stopped and restarted after 3 days (Hutchison & Zeleznik, 1985). The reason for this difference between macaques, in which gonadotrophin secretion is transitorily suppressed by an LHRH antagonist, and macaques in which exogenous LHRH is withdrawn for 3 days and then restored is probably related to the differences in pituitary output of LH during recovery from the LHRH antagonist and re-initiation of LHRH pulses. In the latter, the pituitary LH response to restoration of LHRH is above normal (Hutchison & Zeleznik, 1985). Presumably, while LH is not released when the LHRH pulses are stopped, synthesis continues for some time. The stored hormone is then released in response to the large pulse of exogenous LHRH. In contrast, the recovery period from LHRH antagonist is likely to be gradual as the endogenous LHRH combines with LHRH receptors, the availability of which will be governed in part by the rate of metabolism of the antagonist. Detailed analysis of LH pulse frequency during the recovery period was not possible in the macaques. However, studies in the ram show that after a single injection of LHRH antagonist, the recovery period is associated with a gradual return of LH pulses which are also of increased frequency, presumably due to a response of the LHRH hypothalamic pulse generator to withdrawal of negative feedback (Lincoln & Fraser,1987;1990). Since the frequency of LHRH pulses is thought to be under the influence of negative feedback from progesterone (Ferin et al., 1984), a similar response may occur after LHRH antagonist administration during the luteal phase in the macaque. It is possible that the recovery of apparently normal

progesterone secretion in the lesioned animals is a response to the highly favourable elevated level of LH in the blood while the poor recovery after LHRH antagonist is due to the gradual re-initiation of LH secretion. While the extent of "carry over" during the recovery period may be lessened by reducing the dose of LHRH antagonist, experience with lower doses of this antagonist indicates that suppression of gonadal function is not sustained for a 24h period. Perhaps the use of an antagonist with a more rapid clearance rate may provide a more suitable model.

It appears that while the use of LHRH antagonists provides a non-invasive method for studying the gonadotrophic dependence of ovarian function in experimental animals and in women, their potential in creating a 'window' of suppression followed by a return to normal pituitary-ovarian function may be limited. It is likely that return of gonadotrophin secretion during the recovery period is gradual and may be subject to individual variation. Although the studies of Hutchison & Zeleznik, (1985) imply that the corpus luteum retains a normal function after a 3 day suppression of gonadotrophin secretion, this may have been due to a marked unphysiological increase in LH output on re-initiation of LHRH administration, which would promote a return of progesterone secretion. Further work is required to obtain a more precise control of endogenous gonadotrophin secretion during treatment with LHRH antagonists. The recent report that exogenous LHRH administration can result in uniform pulses of LH in ovariectomized monkeys treated with an LHRH antagonist (Leal et al., 1989), suggests that such an approach may be developed as a suitable model.

Part II. Investigation into the control of Inhibin production *in vitro* using a human luteal cell culture system.

3.2.1 Introduction

To complement the *in vivo* experiments in the stumptailed macaque, a cell culture system was established to investigate if human luteal cells in culture secreted inhibin. The aim of the following set of experiments was firstly, to establish a monolayer culture system which would support the growth of steroid-secreting human luteal cells and secondly to investigate if human luteal cells in culture secreted inhibin, and finally to examine the control of inhibin secretion *in vitro* by administration of hCG.

3.2.2 Materials and Methods

3.2.2.1 Subjects and tissue dating

Corpora lutea at early (1-4 days since ovulation), mid (5-9 days since ovulation) and late (10-14 days since ovulation) stages of the luteal phase were obtained from patients undergoing hysterectomy. None of the patients had received hormonal therapy for three months prior to surgery and informed consent was obtained from all subjects. Ethical approval was obtained from Lothian Area Ethical Committee, Reproductive Medicine Subcommittee, Edinburgh. The stage of the menstrual cycle was determined from the measurement of urinary LH concentrations collected daily for two weeks prior to surgery, histological appearance of endometrial sections obtained during surgery (Noyes et al., 1950; Li et al., 1988) and the date of the last menstrual period.

3.2.2.2 CL enucleation

Corpora lutea were enucleated at the time of surgery by Dr. Peter Illingworth. A circular incision was made through the superficial ovarian capsule and around the circumference of the corpus luteum. The corpus luteum was then enucleated by blunt separation from the surrounding ovarian stroma. The tissue was collected into a sterile jar containing ice-cold phosphate buffered saline (PBS) (Dulbecco's formula, modified, ICN Flow Ltd., Rickmansworth, Herts.) which was then placed in ice for transportation to the laboratory. Adherent connective tissue, epithelium and blood clots were removed using a dissecting microscope and the corpus luteum was then weighed. After rinsing with fresh PBS, the tissue was minced into small fragments using sterile scalpel blades.

3.2.2.3 Cell Dispersion

Human luteal cells were dispersed by a method similar to that described by Fisch et al. (1989). The culture medium used throughout the experiments was Medium 199 containing Earle's salts, 25mM Hepes buffer and 2mM L-glutamine [with added donor calf serum (5% v/v), streptomycin (5000µg/ml) and penicillin (5000IU/ml) from Flow Laboratories]. The procedure for the dispersal of luteal cells was performed in all experiments as follows:

The tissue fragments were added to a sterile glass vial with 5 mls of culture medium containing 0.1% w/v collagenase (type 1A, 570 units/mg solid), and 0.001% DNase (type IV, 2200 Kunitz Units/mg protein) and 0.1% bovine serum albumin (BSA) (all from Sigma Chemical Co. Ltd.). The vial was incubated in a shaking water bath (160

cycles/min) for 30 min at 37°C. Following gentle pipetting of undigested fragments using dispersal pipettes of varying sizes, the suspended cells were collected using a short form glass pipette and fresh enzyme solution replaced in the glass vial. The incubation and cell dispersal procedure was then repeated and suspensions from both incubations pooled. The pool was then filtered through four layers of 12 ply surgical gauze (Smith & Nephew Medical Ltd., Hull) and collected into a 15ml plastic test-tube. The tube was centrifuged at 100xg for 5 min, the resulting pellet re-suspended in a further 5 mls of culture medium/enzyme solution and re-centrifuged. The re-suspension and re-centrifugation step was repeated once more and the cells then resuspended in 1 ml of culture medium. Cell viability was then determined using trypan blue exclusion test in a haemocytometer. This was found to be ≥ 95% in all experiments.

3.2.2.4 Density gradient fractionation

Two Percoll gradients (30% v/v) were then prepared as follows: 1 ml of medium 199 x 10 (Gibco) was added to 9 ml of Percoll (Pharmacia, Uppsala, Sweden), mixed well and further diluted with 23 ml of culture medium. 10 ml of Percoll /culture medium solution was added to two Percoll gradient tubes (Pharmacia) and centrifuged for 30 min at 30,000xg in an ultracentrifuge (Beckman model J2-21) in an angle head rotor (Beckman model JA-20). The cell suspension (1ml) was layered onto one of the gradients and a suspension of marker beads of density ranging from 1.017g/ml to 1.141g/ml (Pharmacia) was applied to the other gradient. This ensured that cells of the same density were used consistently throughout all experiments. The loaded gradients were centrifuged at 800 x g for 20 min.

Following centrifugation, three visible bands of cells appeared as shown diagrammatically in figure 3.4. Red blood cells had a density of 1.14 g/ml, connective tissue at 1.01g/ml and luteal cells at approximately 1.05 g/ml . An 18-G lumbar puncture needle was inserted into the gradient containing the separated cell populations and twenty 0.5 ml fractions were collected into sterile diluting tubes by aspiration. Cells numbers and viability were assessed as before in each of the fractions. Luteal cells were usually concentrated in fractions 3-7 which were pooled, diluted in culture medium to the appropriate dilution and plated in Corning multi-well culture plates (Corning Glass Works, Corning, New York, U.S.A.) according to the particular experiment. All plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. A summary of the method is shown in figure 3.5.

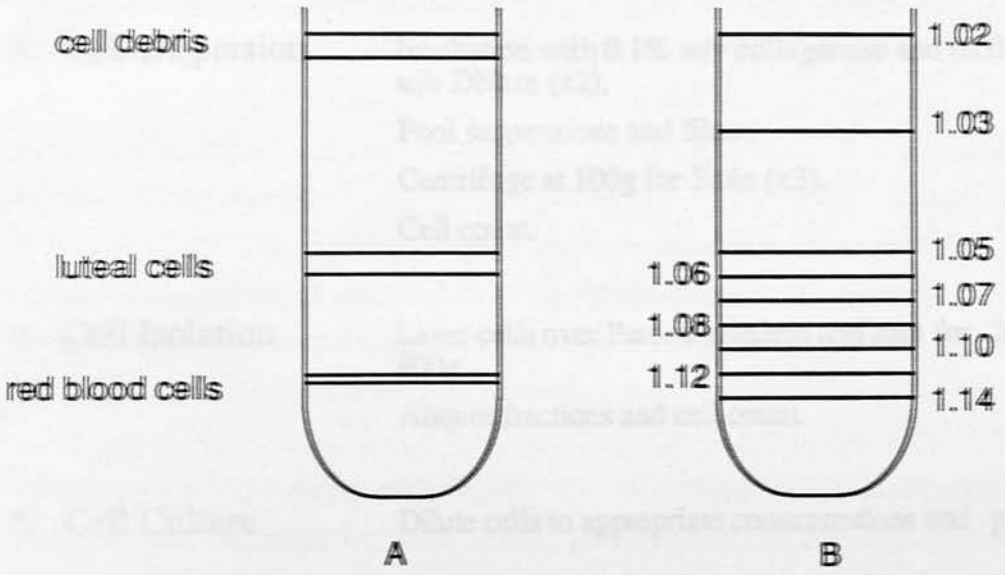


Fig. 3.4. A diagrammatic representation of the appearance of (A) dispersed luteal cells and (B) density marker beads following centrifugation on a Percoll gradient. Red blood cells have a density of approximately 1.14g/ml, connective tissue has a density of approximately 1.01g/ml and luteal cells have a density of approximately 1.05g/ml.

Protocol for Luteal Cell Culture

1. Luteectomy	Enucleation of corpus luteum.
2. Dissection	Removal of connective tissue and blood clots
3. Cell Dispersion	Incubation with 0.1% w/v collagenase and 0.001% w/v DNase (x2). Pool suspensions and filter. Centrifuge at 100g for 5min (x3). Cell count.
4. Cell Isolation	Layer cells over Percoll gradient and spin for 20min at 800g Aliquot fractions and cell count.
5. Cell Culture	Dilute cells to appropriate concentrations and plate.

Fig. 3.5. Summary of method for dispersion and isolation of human luteal cells.

3.2.2.5 Radioimmunoassays

The concentrations of oestradiol, progesterone and inhibin in culture medium were measured as described in chapters 2 and 4, except that the standards for these assays were diluted in culture medium.

3.2.2.6 Statistical analyses

Where appropriate, data were subjected to one- or two- way analyses of variance with log transformation to reduce heterogeneity of variance and subsequently examined using Newman-Keuls test (ANOVA; Clear Lake Research).

3.2.3 Results

3.2.3.1 Experiment 1

The aims of the first experiment were firstly, to establish the method for obtaining enriched populations of steroid-producing human luteal cells, secondly to determine which of the fractions contained the steroid-producing cells and finally, to investigate the production of steroids over a 96h period.

Luteal cells were isolated and dispersed as described in 3.2.2. Sixteen cellular fractions were aspirated from the Percoll gradient and diluted to 3mls with culture medium. 250µl of cell suspension from each fraction was added to Corning multi-well culture plates in triplicate and the following treatments were set up for each fraction in triplicate:

(i) no treatment (control)

(ii) 15ng/ml hCG (CR 125 hCG, specific biologic activity 11 900 IU/mg from NIDDK and NHPP, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.)

(iii) 10^{-6}M testosterone (Sigma Chemical Co. Ltd.)

(iv) 15ng/ml hCG + 10^{-6}M testosterone.

Culture medium was changed at 48h and 96h and stored at -20°C until assayed for progesterone and oestradiol. This experiment was repeated three times using different corpora lutea.

3β-hydroxysteroid dehydrogenase cytochemistry

To further identify which fractions contained the steroidogenic luteal cells, a sample of cells from each fraction was stained cytochemically for the presence of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. The cytochemical reaction utilizes nitro-blue tetrazolium as hydrogen acceptor and dehydroisoandrostenone as a

substrate. Intracellular sites of 3β-HSD activity are demonstrated by the presence of blue granules in the cytoplasm.

The cytochemical procedure was carried out using a modification of the procedure described by Steinberger et al. (1966). Cells from each fraction were plated in triplicate at 50 000 cells/well and incubated for 24h. The culture medium was then removed and the cells washed three times with PBS-BSA. 300µl of 3β-HSD staining solution (0.07M phosphate-buffered saline containing 1mg/ml niacinamide, 6mg/ml β-nicotinamide adenine dinucleotide, 1.5 mg/ml nitro-blue tetrazolium and 100µg/ml dehydroisoandrosterone) was added to each well and incubated for 2h at 37°C. Control wells were set up containing 3β-HSD solution without substrate to demonstrate the specificity of the staining reaction. All chemicals were obtained from Sigma Chemical Co. Ltd. This experiment was carried out on three separate corpora lutea and representative results are shown and described as follows:

Results- Experiment 1

Steroid production in culture.

Both progesterone and oestradiol were produced by the luteal cells throughout the 96h period. The mean progesterone production from sixteen fractions in control and treated wells is shown in figure 3.6 (0-48h) and figure 3.7 (48-96h). The cells with the greatest progesterone producing capacity were concentrated in fraction 4. There was an increase in progesterone secretion during the second 48h period in culture. Oestradiol production from the sixteen fractions is shown in figure 3.8 (0-48h) and figure 3.9 (48-96h). The fractions producing the greatest amount of oestradiol were also in fractions 4. In contrast with progesterone, there was a decline in oestradiol production during the second 48h, regardless of the presence of testosterone as an aromatase substrate.

3β-HSD cytochemistry

Positive staining corresponding to 3β-HSD activity was first detected at 30min in the wells containing cells from fractions 2-9. However by 2h, the greatest intensity of staining was present in the wells containing cells from fractions 2-7, demonstrating that the steroidogenic cells were predominantly located in these fractions.

Steroid response to hCG and testosterone.

To examine the steroid response to treatments more closely, progesterone and oestradiol production during 0-48h and 48-96h from fraction 3 is shown in figure 3.10

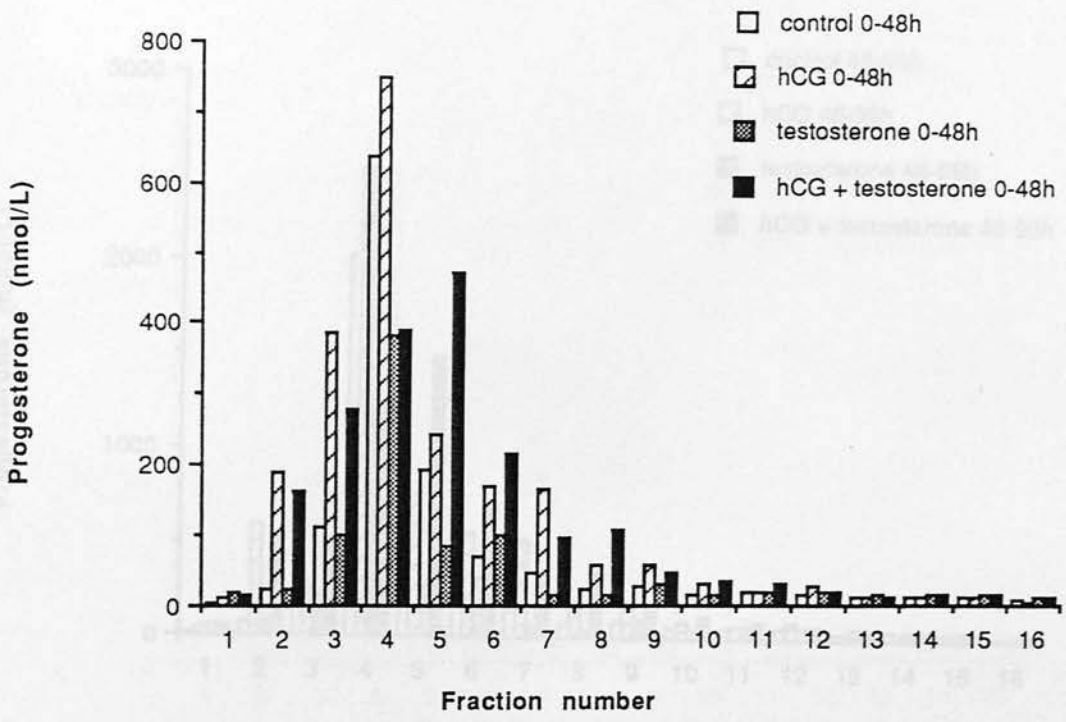


Fig. 3.6. Progesterone production from cellular fractions from 0-48h.

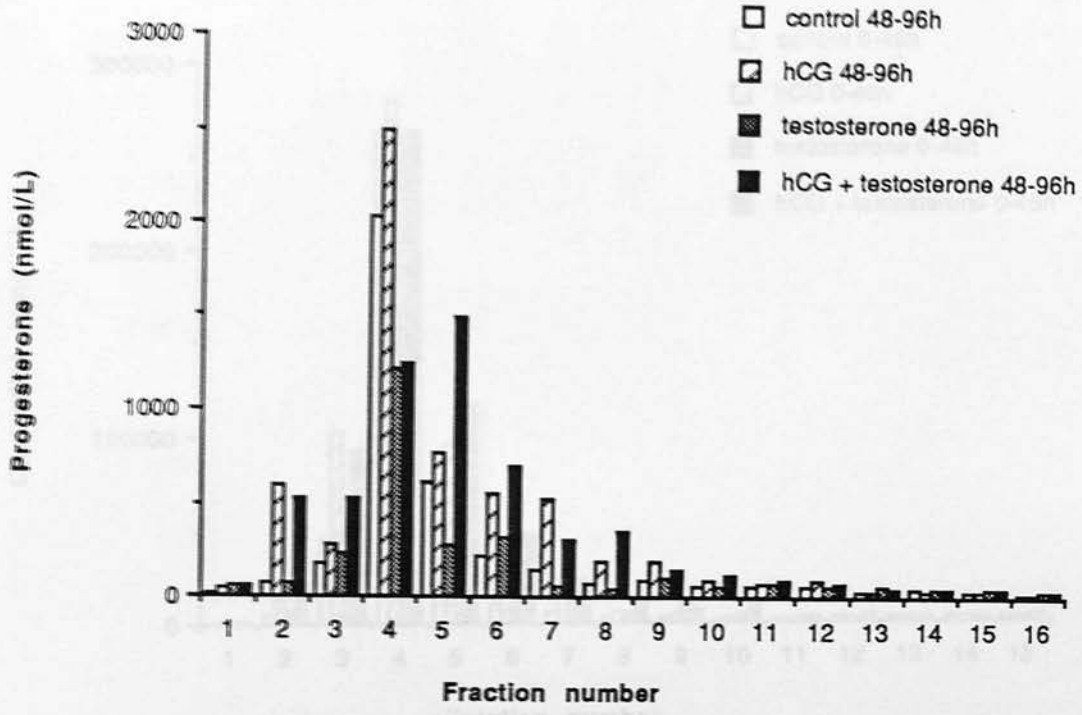


Fig. 3.7. Progesterone production from cellular fractions from 48-96h.

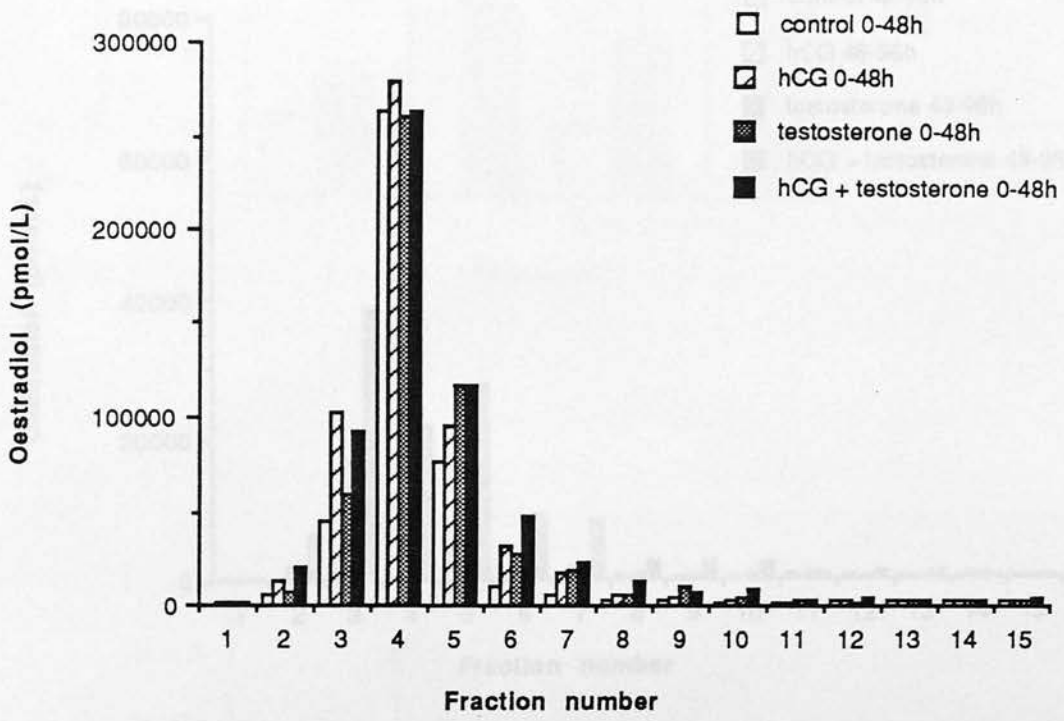


Fig. 3.8. Oestradiol production from cellular fractions from 0-48h.

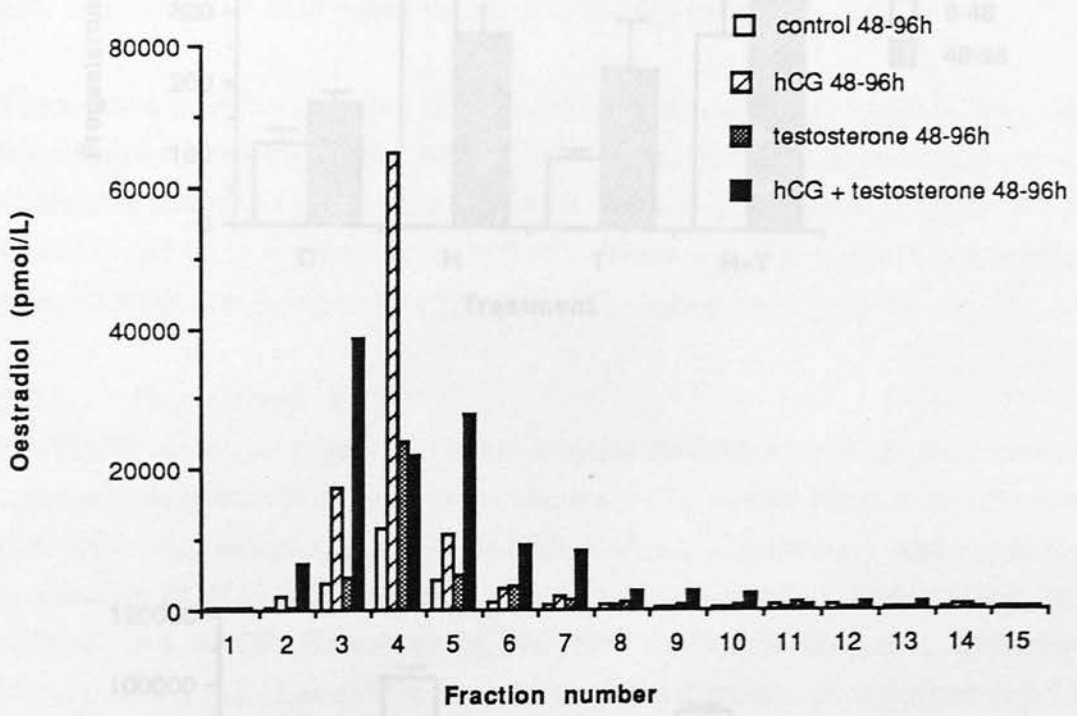
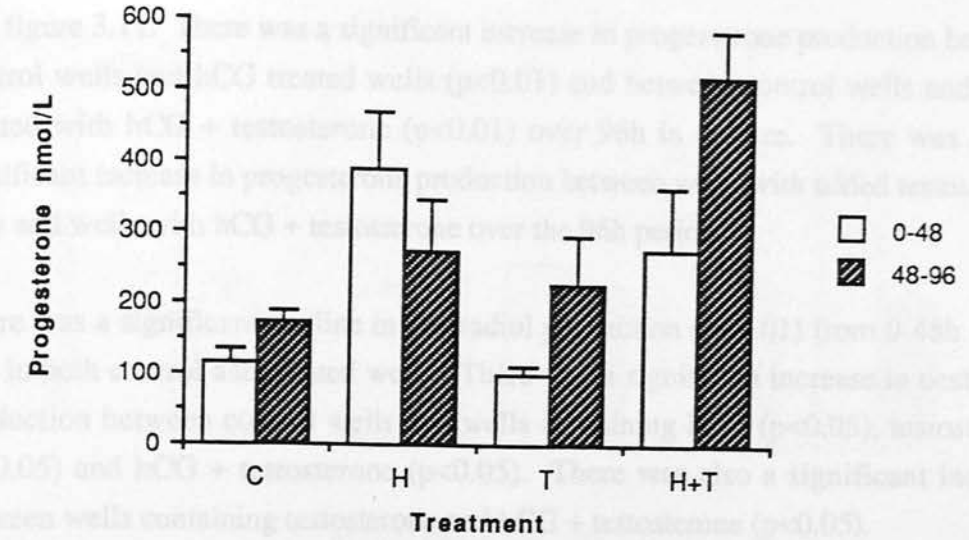
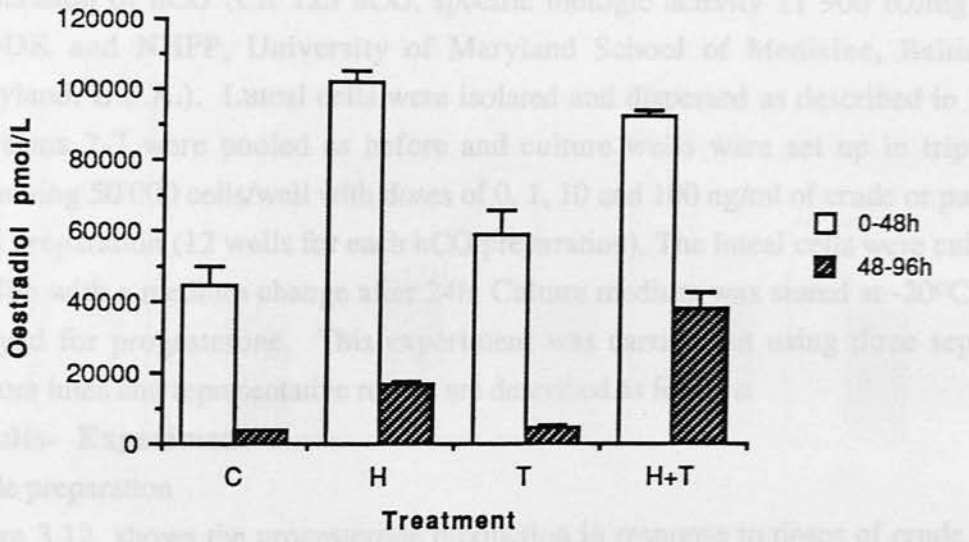


Fig. 3.9. Oestradiol production from cellular fractions from 48-96h.





Progesterone (Fig. 3.10) and oestradiol (Fig. 3.11) production from fraction 3 from 0-48h and 48-96h in response to control (C), hCG (H), testosterone (T) and hCG + testosterone (H+T) treated wells.

and figure 3.11. There was a significant increase in progesterone production between control wells and hCG treated wells ($p<0.01$) and between control wells and wells treated with hCG + testosterone ($p<0.01$) over 96h in culture. There was also a significant increase in progesterone production between wells with added testosterone only and wells with hCG + testosterone over the 96h period.

There was a significant decline in oestradiol production ($p<0.01$) from 0-48h to 48-96h in both control and treated wells. There was a significant increase in oestradiol production between control wells and wells containing hCG ($p<0.05$), testosterone ($p<0.05$) and hCG + testosterone ($p<0.05$). There was also a significant increase between wells containing testosterone and hCG + testosterone ($p<0.05$).

3.2.3.2 Experiment 2

The aim of the second experiment was to examine the differences in responsiveness of luteal cells in culture to different preparations of hCG, a crude preparation (Chorulon [1500IU/vial], Intervet U.K., Ltd., Milton Road, Cambridge) and a purified preparation of hCG (CR 125 hCG, specific biologic activity 11 900 IU/mg from NIDDK and NHPP, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.). Luteal cells were isolated and dispersed as described in 3.2.2. Fractions 2-7 were pooled as before and culture wells were set up in triplicate containing 50 000 cells/well with doses of 0, 1, 10 and 100 ng/ml of crude or purified hCG preparation (12 wells for each hCG preparation). The luteal cells were cultured for 48h with a medium change after 24h. Culture medium was stored at -20°C until assayed for progesterone. This experiment was carried out using three separate corpora lutea and representative results are described as follows:

Results- Experiment 2

Crude preparation

Figure 3.12 shows the progesterone production in response to doses of crude hCG over 48h. There was a significant increase in progesterone production between control wells and wells containing 10ng/ml and 100ng/ml hCG ($p<0.05$). There was a significant decline in progesterone production between 0-24h and 24-48h in the wells containing 100ng/ml hCG.

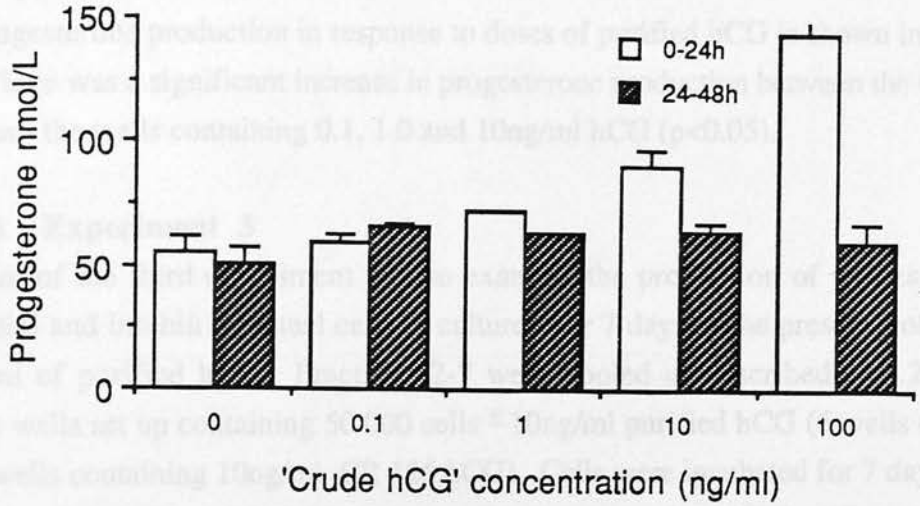


Fig. 3.12. Progesterone production by luteal cells in culture from 0-24h and 24-48h in response to 0-100ng/ml of a crude preparation of hCG (Chorulon).

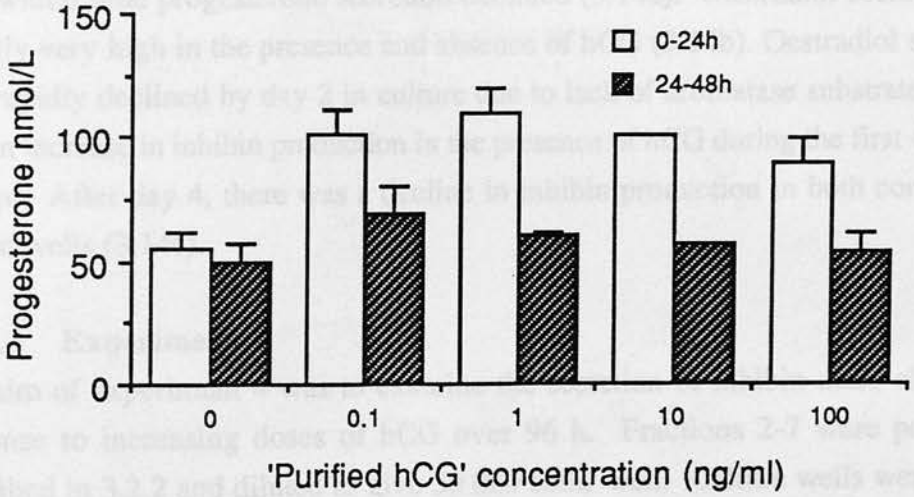


Fig. 3.13. Progesterone production by luteal cells in culture from 0-24h and 24-48h in response to 0-100ng/ml of a pure preparation of hCG (CR 125).

Pure preparation

The progesterone production in response to doses of purified hCG is shown in figure 3.13. There was a significant increase in progesterone production between the control wells and the wells containing 0.1, 1.0 and 10ng/ml hCG ($p<0.05$).

3.2.3.3 Experiment 3

The aim of the third experiment was to examine the production of progesterone, oestradiol and inhibin by luteal cells in culture over 7 days in the presence of 0 and 10ng/ml of purified hCG. Fractions 2-7 were pooled as described in 3.2.2 and culture wells set up containing 50 000 cells $\pm$ 10ng/ml purified hCG (6 wells control and 6 wells containing 10ng/ml CR 125 hCG). Cells were incubated for 7 days with culture medium changed daily. Culture medium was stored at -20°C until assayed for progesterone, oestradiol and inhibin. This experiment was carried out on three separate corpora lutea and representative results are described as follows:

Results- Experiment 3

In the presence of hCG, there was an increase in progesterone secretion until day 4 in culture. Progesterone secretion then remained relatively constant until day 6 in culture after which time progesterone secretion declined (3.14a). Oestradiol secretion was initially very high in the presence and absence of hCG (3.14b). Oestradiol secretion then rapidly declined by day 2 in culture due to lack of aromatase substrate. There was an increase in inhibin production in the presence of hCG during the first 4 days in culture. After day 4, there was a decline in inhibin production in both control and treated wells (3.14c).

3.2.3.4 Experiment 4

The aim of experiment 4 was to examine the secretion of inhibin more closely in response to increasing doses of hCG over 96 h. Fractions 2-7 were pooled as described in 3.2.2 and diluted to give 50 000 cells/ well. Culture wells were set up (n=6 per treatment) containing 0, 0.01, 0.1, 1, and 10 ng/ml hCG (CR 125 hCG). Culture medium was changed at 48 and 96h and stored at -20°C until assayed for progesterone and inhibin.

Results- Experiment 4

A significant increase in progesterone production between control and 0.01, 0.1, 1 and 10 ng/ml hCG ($p<0.05$) was observed (fig. 3.15). A significant increase in inhibin production between control and hCG treated wells was also observed at all

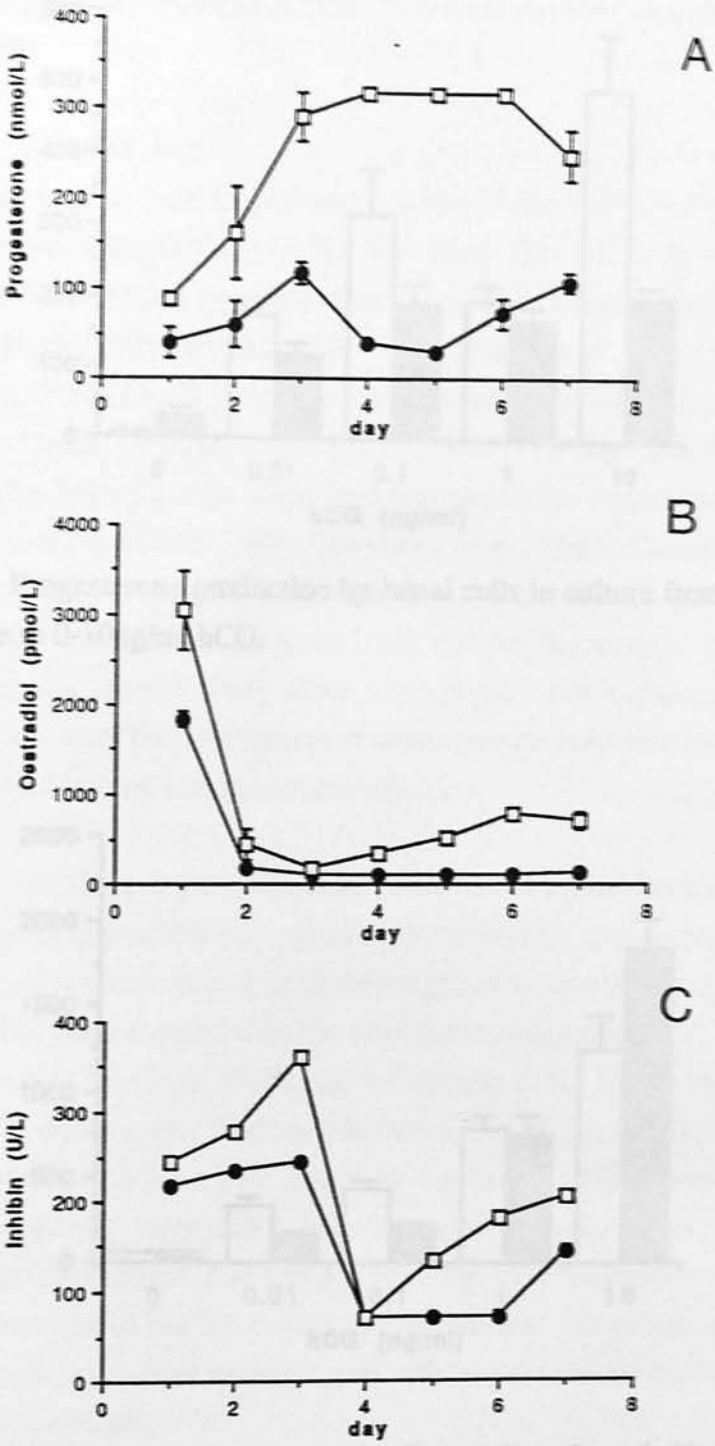


Fig. 3.14. Progesterone (A), oestradiol (B) and inhibin (C) concentrations from luteal cells in culture over 7 days in response to either control (●) or 10ng/ml hCG (□).

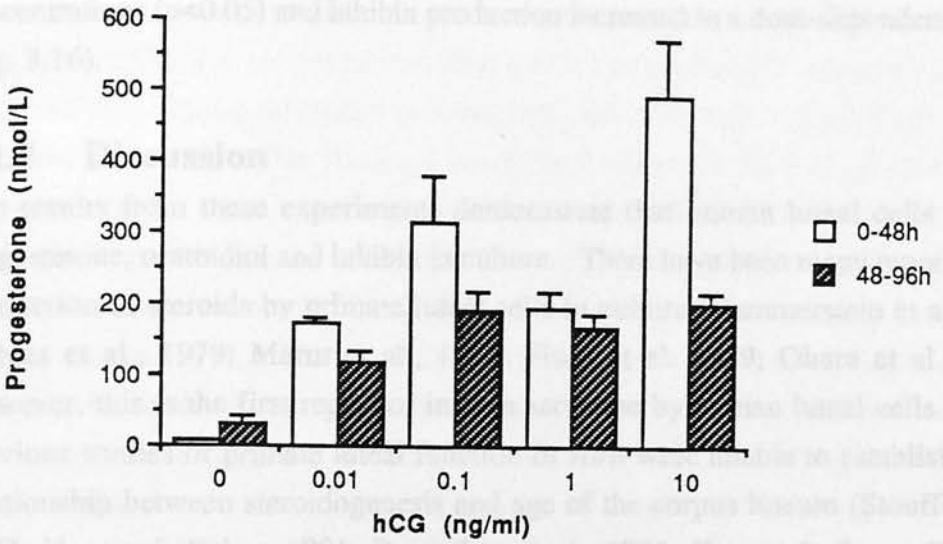


Fig. 3.15. Progesterone production by luteal cells in culture from 0-48h and 48-96h in response to 0-10ng/ml hCG.

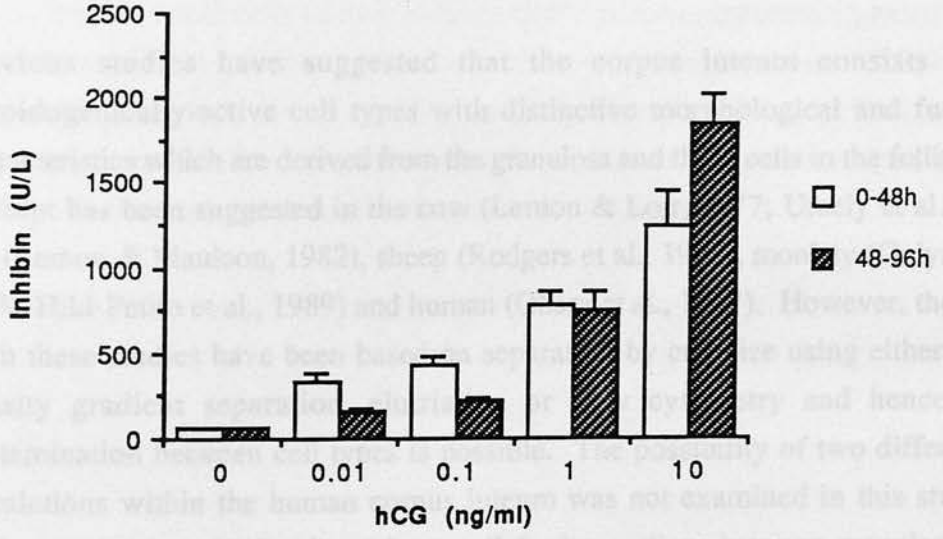


Fig. 3.16. Inhibin production by luteal cells in culture from 0-48h and 48-96h in response to 0-10ng/ml hCG.

concentrations ($p<0.05$) and inhibin production increased in a dose-dependent manner (fig. 3.16).

3.2.4 Discussion

The results from these experiments demonstrate that human luteal cells produce progesterone, oestradiol and inhibin in culture. There have been many reports on the production of steroids by primate luteal cells in culture (Hammerstein et al., 1964; Gulyas et al., 1979; Marut et al., 1983; Fisch et al. 1989; Ohara et al., 1987). However, this is the first report of inhibin secretion by human luteal cells *in vitro*. Previous studies of primate luteal function *in vitro* were unable to establish a clear relationship between steroidogenesis and age of the corpus luteum (Stouffer et al., 1977; Hunter & Baker, 1981; Dennefors et al., 1982; Casper & Cotterell, 1984). However, Fisch et al., (1989) demonstrated that the steroidogenic capacity of human luteal cells diminished progressively during the course of the luteal phase. Unfortunately, in this study there were insufficient numbers of corpora lutea at different stages of the luteal phase to investigate the relationship between the maturity of the corpus luteum and hormone production.

Previous studies have suggested that the corpus luteum consists of two steroidogenically-active cell types with distinctive morphological and functional characteristics which are derived from the granulosa and theca cells in the follicle. This concept has been suggested in the cow (Lemon & Loir, 1977; Ursely et al., 1981), pig (Lemon & Mauleon, 1982), sheep (Rodgers et al., 1984), monkey (Gulyas et al., 1979, Hild-Petito et al., 1989) and human (Ohara et al., 1987). However, the results from these studies have been based on separation by cell size using either Percoll density gradient separation, elutriation or flow cytometry and hence cross-contamination between cell types is possible. The possibility of two different cell populations within the human corpus luteum was not examined in this study and further work is required to investigate cellular interactions between granulosa-lutein and theca-lutein cells.

Previous studies examining the production of inhibin secretion *in vitro* have focussed on granulosa cells. Bioactive inhibin is produced by human granulosa cells *in vitro* and its release is stimulated by FSH and LH (Tsonis et al., 1987b). The stimulation of inhibin production by granulosa cells from marmoset and women by FSH is further enhanced by both testosterone and oestradiol (Hillier et al., 1989). Furthermore, in a detailed study by Hillier et al. (1991a) the capacity of granulosa cells to produce

immunoreactive inhibin *in vitro* was demonstrated to increase with follicular maturity. FSH but not LH was shown to stimulate inhibin production by immature granulosa cells whereas during advanced preovulatory development, inhibin production is responsive to LH. The findings from the studies in Part II of this chapter demonstrated that cellular production of inhibin *in vitro* is directly stimulated by hCG. The differing responses obtained using the crude and purified hCG preparations, although equivalent units of each were added to the cells in culture, may be explained by the possibility that the preparations were standardised in different bioassay systems. The inference that LH is the gonadotrophin controlling inhibin secretion was also suggested in chapter 1 where hCG maintained both inhibin and progesterone concentrations when administered during the mid-luteal phase and in Part I of this chapter, where hCG administration could overcome LHRH antagonist induced suppression of inhibin and progesterone during the mid-luteal phase.

When the production of inhibin was examined in culture over 7 days, there was a decline in inhibin production after 3 days whereas progesterone secretion was maintained until day 6. This may suggest the depletion of a factor required for inhibin production by luteal cells or may indicate that a different mechanism exists for LH-dependent inhibin production in comparison with progesterone. This has been suggested previously in an *in vivo* study by Nakajima et al. (1990) where no correlation was found between the pulsatile profiles of peripheral progesterone and inhibin concentrations in women during the mid-luteal phase.

Chapter 4

Inhibin secretion following LHRH agonist treatment.

Part 1. LHRH agonist treatment and ovarian hyperstimulation during the follicular phase in the macaque.

4.1.1 Introduction

Ovarian hyperstimulation by exogenous gonadotrophins is widely used both in women and in monkeys to obtain large numbers of oocytes for use in in vitro fertilization programmes (Boatman et al., 1986; McLachlan et al., 1986c; Messinis & Templeton, 1987, 1989; Glasier et al., 1988; Tsonis et al., 1988). There are indications that stimulation of the ovary in this way can induce alterations in the normal positive feedback relationship between oestradiol and the pituitary. The incidence of an LH surge is reduced and its magnitude is always attenuated. It has been suggested that this adverse effect is brought about by a non-steroidal product of the developing follicles (Littman & Hodgen, 1984; Schenken & Hodgen, 1983; Stillman et al., 1983; Messinis & Templeton, 1987, 1989).

The aim of this study was to use exogenous FSH to induce follicular hyperstimulation in the stumptailed macaque. The effects of this treatment on immunoreactive inhibin concentrations in peripheral blood were compared to the situation found during the normal cycle. Before FSH treatment, follicular development and ovulation were suppressed by inducing pituitary desensitization with an implant of an agonist of luteinizing hormone-releasing hormone (LHRH) (Fraser et al., 1987b; Fraser & Lunn, 1989). This permitted synchronization of the timing of the stimulatory treatment and restricted the observations to follicular development by avoiding the induction of ovulation which is known to be associated with an elevation in inhibin concentrations.

4.1.2 Materials and Methods

4.1.2.1 Control cycles

Ten adult female stumptailed macaques *(Macaca arctoides)* weighing 9-14 kg were used. The animals had exhibited regular menstrual cycles with normal luteal phases, as determined by hormonal estimations 3 times per week, fulfilling the criteria described previously in chapter 2. Blood samples (4 ml) were collected daily by femoral

venepuncture without anaesthesia, as described in chapter 2, beginning during the early follicular phase and continuing until the end of the cycle. Samples were centrifuged at 1000 g for 20 min, the serum divided into two aliquots and stored at -20°C until assayed for progesterone, oestradiol-17β, LH and inhibin.

4.1.2.2 LHRH agonist implant

Four adult female stump-tailed macaques with regular menstrual cycles each received a single implant containing 3 mg of buserelin, ([D-Ser (tBu)^6,Pro^9-NHEt] LHRH 1-9 (Dr J. Sandow and H. Siedel, Hoechst AG, Frankfurt, FRG). The implants measured 0.8 x 0.12 cm and were made of slowly biodegradable polylactic / glycolide (molar ratio 75:25) sterilized by exposure to 1.2Mrad of gamma radiation. The LHRH agonist implant was administered during the early follicular phase of the cycle. The macaques were lightly sedated using ketamine hydrochloride (10mg/kg) (Vetalar: Parke, Davis and Co., Pontypool, Gwent) and the implant was injected s.c. in the lateral region of the abdominal wall using a sterile applicator by Dr.Hamish Fraser. This was a very simple procedure with no sutures required. Blood samples were collected at least 3 times per week after implant administration.

4.1.2.3 LHRH test

At 1week before the treatment with FSH, an LHRH test was performed by i.v. injection of 50 μg LHRH (Gonadorelin: Ayerst Laboratories Ltd., Andover, Hants, U.K.). This was procedure was carried out by Dr. Hamish Fraser. Blood samples were collected at 0, 30 and 60 min. The test was repeated on the final day of FSH treatment. Results were compared with those obtained after carrying out the same LHRH test in 6 control animals during the early follicular phase of the normal cycle.

4.1.2.4 FSH treatment

At 8 weeks after receiving the implant, the macaques were treated with FSH (Metrodin, Serono Laboratories, Welwyn Garden City, Herts, UK) in 1 ml 0.9% w/v sodium chloride solution i.m. once daily for 9 days (75 i.u. on Day 0, 35 i.u. on Days 1-8). This was carried out by Dr. Hamish Fraser and the staff at the Primate Unit.

4.1.2.5 Assays

Serum LH concentrations during the LHRH tests were determined using an *in vitro* bioassay by Dr.Stephen Lunn based on the production of testosterone by dispersed mouse Leydig cells as described previously (Fraser et al., 1986). Sensitivity of the assay was 1.0 ng LH NICHHD rhesus monkey pituitary standard RP-1 /ml. Inter- and

intra-assay coefficients of variance were 15 and 11% respectively. To determine the day of the preovulatory LH surge, serum LH and progesterone were measured by radioimmunoassay as described in chapter 2. Inhibin concentrations were measured by the heterologous RIA described in chapter 2.

Oestradiol 17-β assay

General radioimmunoassay procedure was followed as described in chapter 2. Serum oestradiol concentrations were measured by a specific RIA following diethyl ether extraction of serum (Glasier et al., 1989). The oestradiol-^{125}I iodohistamine tracer was prepared by Mr.George Johnston essentially as described by Hunter et al., 1975, with the modified extraction procedure which increased the recovery of radiolabelled product after iodination (Hillier & Read, 1975). The reference standard used was 17β-oestradiol (Sigma Chemical Co. Ltd.) (E8875) at concentrations of 2.5-320 pg/0.1ml. A specific sheep anti-oestradiol antibody (BW 26/9/80) (provided by Dr.R.Webb, ABRO, Midlothian) was used at an initial dilution of 1:2,000,000. Sensitivity of the RIA was 20pg/ml and inter- and intra-assay coefficients of variation were 11% and 5% respectively.

Assay buffer

Phosphate buffered saline with gelatine (PBGS) pH 7.4

NaCl	9g/L
Na_2HPO_4	8.6g/L
$Na_2PO_42H_2O$	6.08g/L
Gelatin	1.0g/L
Thiomersalate	0.1g/L

All chemicals were purchased from BDH Chemicals Ltd.

Sample Extraction

300µl of each sample was aliquoted singly. 3mls of fresh diethyl ether (BDH Chemicals Ltd., Poole) was added to each tube and mixed for 5 min. The organic and aqueous phases were separated by freezing in a dry ice/methanol mixture, supernatants were decanted and evaporated to dryness on a heated block. Standards (100µl) were aliquoted and treated in a similar manner.

Calculation of extraction recovery

150µl of recovery tracer (2,4,6,7-^{3}H oestradiol in toluene/ether (9:1), Amersham International, P.L.C.) was added to 300µl of a macaque quality control pool in

duplicate. Diethyl ether (3ml) was then added to each of the duplicates which were then treated as before. The recovery samples were reconstituted with 200µl of PBGS and incubated overnight at 4°C. Scintillation fluid (3ml) (Ecoscint, National Diagonistics, Aylesbury, Bucks.) was then added to 100µl of recovery sample and counted for 1 min in a β counter (Rack Beta 'Primo' Liquid Scintillation Counter, LKB Wallac, Turku, Finland). The cpm were then doubled and % recovery calculated.

Radioimmunoassay procedure

Day 1. 200µl of antibody and 200µl tracer were added to each extracted assay tube. Assay was then incubated for 2 hours at room temperature.

Day 2. 500µl of 1.25% dextran (T70, Pharmacia)/1.25% charcoal(activated, Sigma Chemical Co., Ltd., Poole) was then added to each tube. All tubes were mixed, placed in ice for 10 min and spun at 3000 rpm for 10min at 4°C. Supernatants were then poured into new assay tubes and counted in a gamma counter as before.

4.1.3 Results

Serum concentrations of oestradiol, progesterone and inhibin, centred around the day of the mid-cycle LH surge (Day 0) in 10 control animals, are shown in figure 4.1(a). Inhibin concentrations were low during the follicular phase in all animals before the onset of a sustained rise from Day 4 or 5 after the LH surge. Levels of inhibin increased to maximal values during the mid-luteal phase before falling as the luteal phase came to an end.

The LHRH agonist implant injection induced a transitory rise in oestradiol and inhibin (fig.4.1(b)). Thereafter, buserelin-implanted animals had low serum concentrations of inhibin, similar to those found during the early-mid follicular phase of the cycle and oestradiol was suppressed to early follicular phase values.

In the 6 control animals receiving an LHRH test during the early follicular phase of the cycle, serum LH concentrations increased from 18.1±0.3 ng/ml at time 0 to 39.3±6.7 and 34.2±4.1 ng/ml at 30 and 60 min respectively. In contrast, in animals treated with LHRH agonist implant and challenged acutely prior to the administration of FSH, serum LH concentrations were around the detection limit of the LH bioassay (1.7 ±0.6 ng/ml) at time 0, rising to 5.8±3.2 ng/ml and 5.1±2.6 ng/ml at 30 and 60 min. This small rise was attributable to a response in one animal (no. 85).

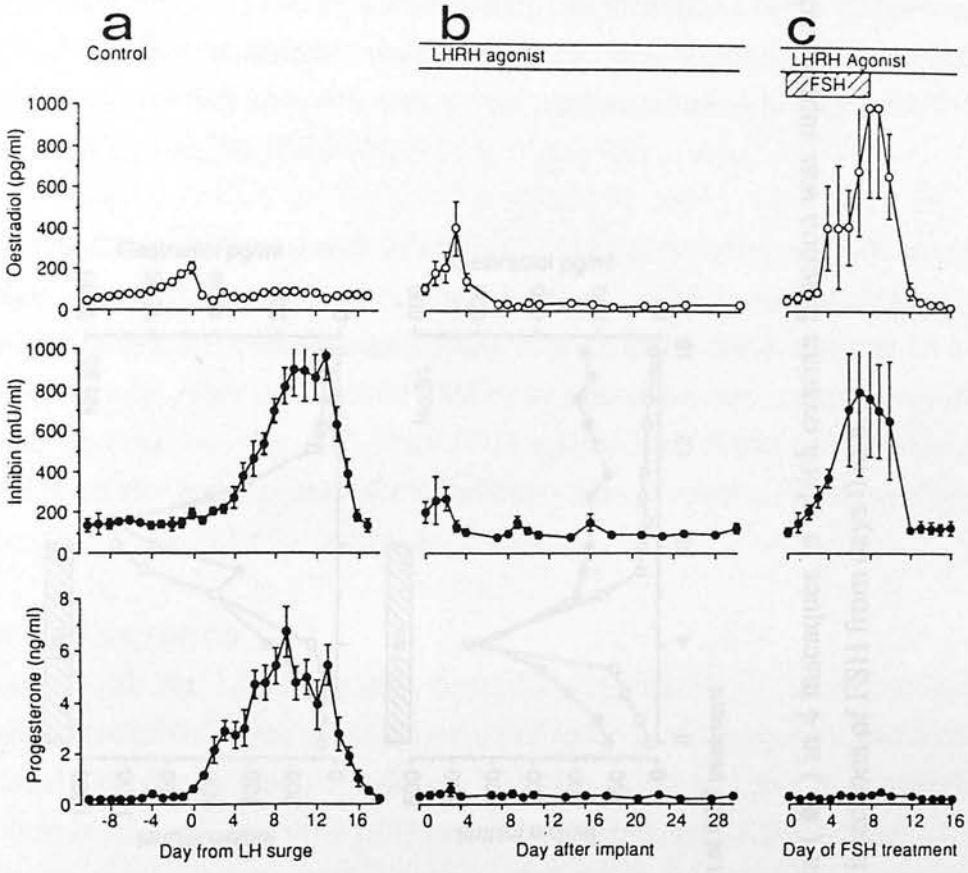


Fig. 4.1. Serum concentrations of progesterone, oestradiol and inhibin in 10 control macaques, data centred around the time of the pre-ovulatory LH surge (day 0), in 4 macaques after treatment with an LHRH agonist implant starting during the early follicular phase and in the same animals after treatment with daily i.m. injections of FSH from days 0-8. Values are plotted as the mean ± S.E.M.

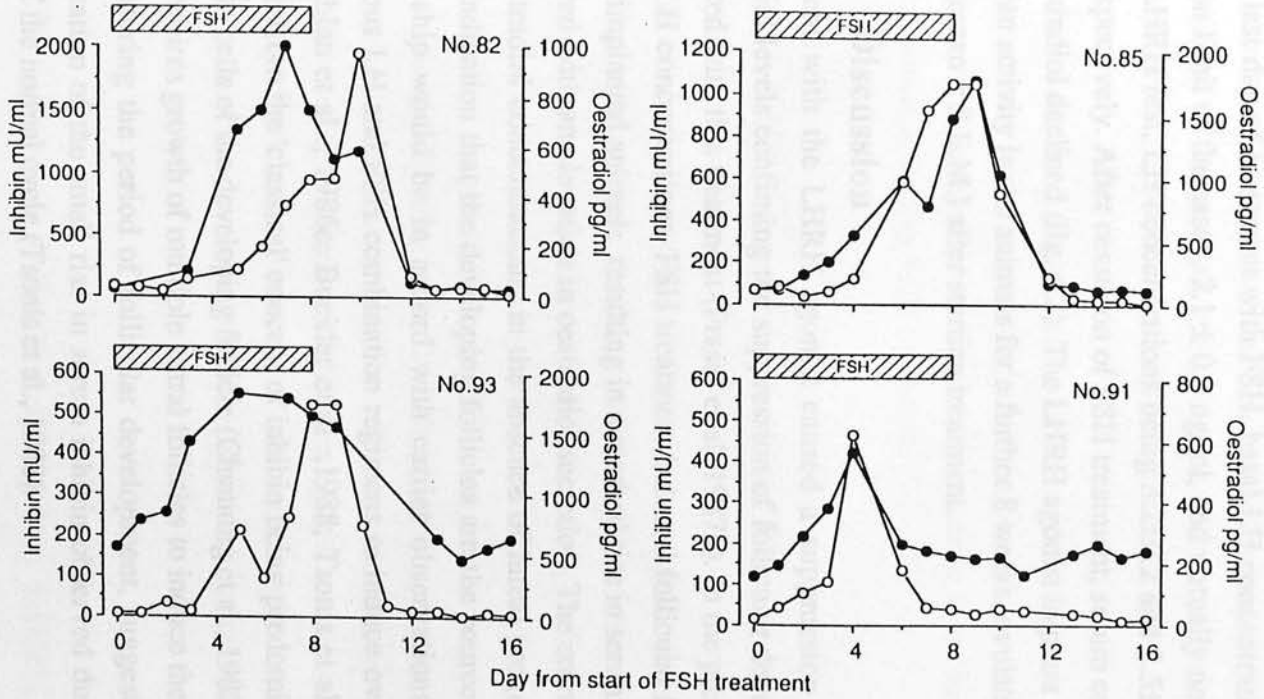


Fig. 4.2. Serum concentrations of oestradiol (O) and inhibin (●) in 4 macaques in which ovarian function was suppressed by an LHRH agonist implant and subsequently treated with daily i.m. injections of FSH from days 0 to 8.

The treatment with FSH resulted in marked rises in serum concentrations of inhibin and oestradiol beginning 3-4 days after starting treatment (fig.4.1(c)). There was considerable individual variation in response as shown in figure 4.2, but the pattern of oestradiol and inhibin response was similar, the correlation between inhibin and oestradiol being r=0.871 ($P< 0.001$).

On the last day of treatment with FSH, basal LH concentration was still around the detection limit of the assay, 2.1 ± 0.2 ng/ml, and virtually no response was obtained to the LHRH test, LH concentrations being 3.2±0.2 and 2.5±0.5 ng/ml at 30 and 60 min, respectively. After cessation of FSH treatment, serum concentrations of inhibin and oestradiol declined (fig. 4.2). The LHRH agonist implant maintained suppression of ovarian activity in the animals for a further 8 weeks, ovulation occurring 19.9 ± 1.3 weeks (mean ± S.E.M.) after starting treatment.

4.1.4 Discussion

Treatment with the LHRH agonist caused a suppression of serum inhibin and oestradiol levels confiming the suppression of follicular development and ovulation associated with this treatment (Fraser et al.,1987b). In the presence of extremely low serum LH concentrations, FSH treatment induced follicular hyperstimulation in the agonist implanted animals, resulting in a marked rise in serum inhibin concentrations associated with an elevation in oestradiol secretion. The correlation between inhibin and oestradiol concentrations, in the absence of luteal progesterone secretion, is a strong indication that the developing follicles are the source of the inhibin. Such a relationship would be in accord with earlier observations in women receiving exogenous LH and FSH combination regimens to induce ovarian hyperstimulation (McLachlan et al ., 1986c; Buckler et al .,1988; Tsonis et al., 1988) and would be expected from the 'classical' concept of inhibin being predominantly a product of the granulosa cells of the developing follicle (Channing et al., 1982). However, it seems that it requires growth of multiple antral follicles to induce the marked rises in serum inhibin during the period of follicular development, suggesting the increase is an amplification of the small rise in serum inhibin observed during the late follicular phase of the normal cycle (Tsonis et al., 1988).

Studies of macaques and women receiving gonadotrophin treatment to induce ovarian hyperstimulation have revealed an attenuation or inhibition of the LH surge, decreased responsiveness to LHRH, and prevention of ovulation (Schenken & Hodgen, 1983; Littman & Hodgen, 1984; Messinis & Templeton, 1987, 1989). It has been

suggested that these inhibitory effects are the result of the production of a gonadotrophin surge inhibitory factor which is peptidic in nature (Stillman et al., 1983). Although it was thought originally that this substance might be inhibin, the demonstration of a substance in extracts of porcine and human follicular fluid which suppressed LHRH-stimulated LH and FSH release was in contrast to the effects of an inhibin preparation which suppressed FSH selectively in an *in vitro* bioassay. Since the substance with these properties also had different physiochemical characteristics from inhibin, this suggested the presence of an additional gonadotrophin surge inhibitory factor (Danforth et al., 1987; Fowler et al., 1989).

In conclusion, it is apparent that a number of substances are pharmacologically elevated in blood by hyperstimulation protocols. These results emphasize the non-physiological nature of the rise in inhibin, resulting from the induction of follicular hyperstimulation following FSH treatment.

4.2
Part II. LHRH agonist implant during luteal phase in the macaque.

The effect of administration of an LHRH agonist implant during the luteal phase in the stumptailed macaque was investigated. Five adult female macaques were implanted with buserelin rods during the mid-luteal phase as described in part I. Blood samples were collected at least three times per week thereafter and samples treated as described in part I. Serum progesterone and inhibin concentrations are shown in figure 4.3. Progesterone and inhibin were initially at luteal phase levels and (similar to administration of implant during the follicular phase) there was a decline in serum concentrations of both hormones following agonist administration, the fall in inhibin being delayed in comparison with progesterone. Serum concentrations of progesterone and inhibin then remained suppressed for the remainder of the sampling period.

4.3
Part III. Recovery Period following LHRH agonist implant in the macaque.

Blood sampling was continued three times per week in LHRH agonist implanted macaques treated in both the follicular and luteal phases (n=5 per group) until the first recovery cycle. The time to return to ovulation varied between individual animals ranging from 85 to 144 days in follicular phase-treated animals and 87 to 161 days in

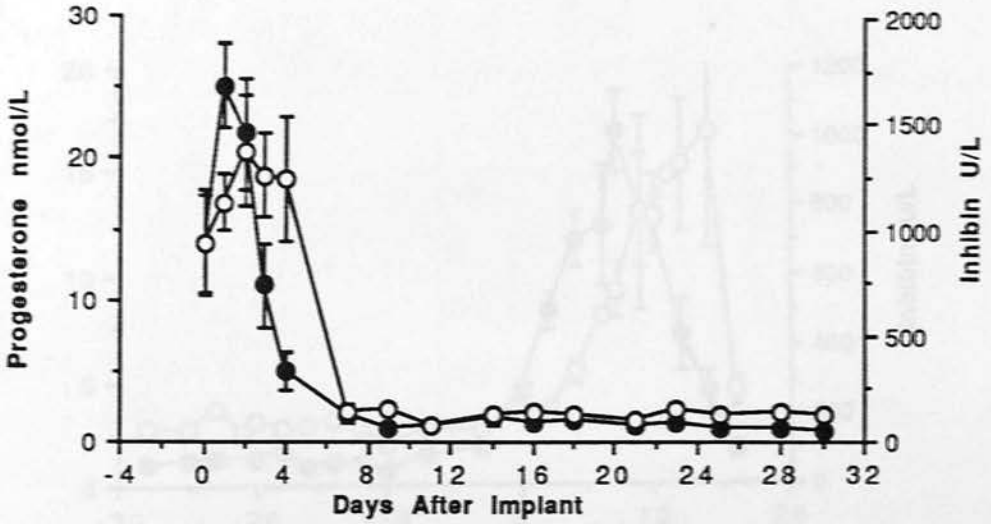


Fig. 4.3. Serum concentrations of progesterone (●) and inhibin (O) in macaques treated with LHRH agonist implant during the luteal phase (n=5). Values are means ± S.E.M.

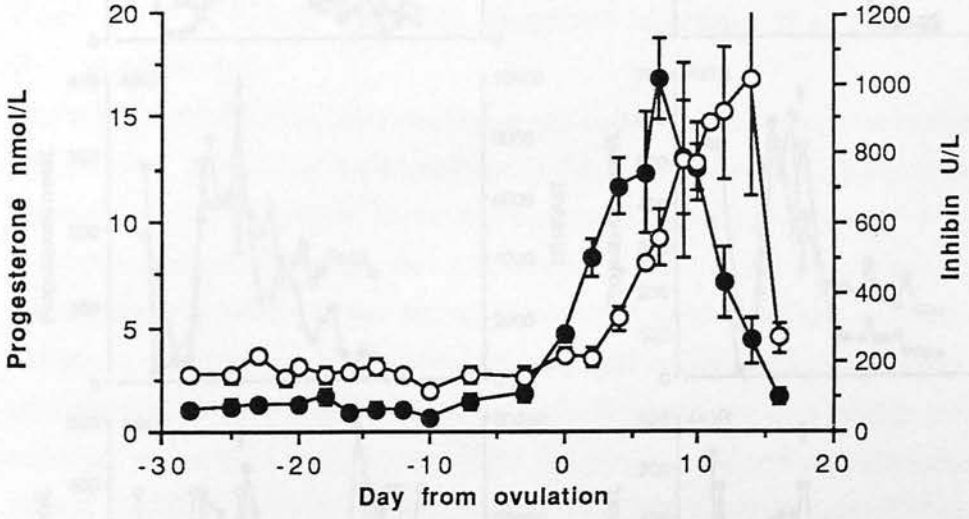


Fig. 4.4. Serum concentrations of progesterone (●) and inhibin (O) in recovery cycle following LHRH agonist implant in 10 macaques. Values are means ± S.E.M.

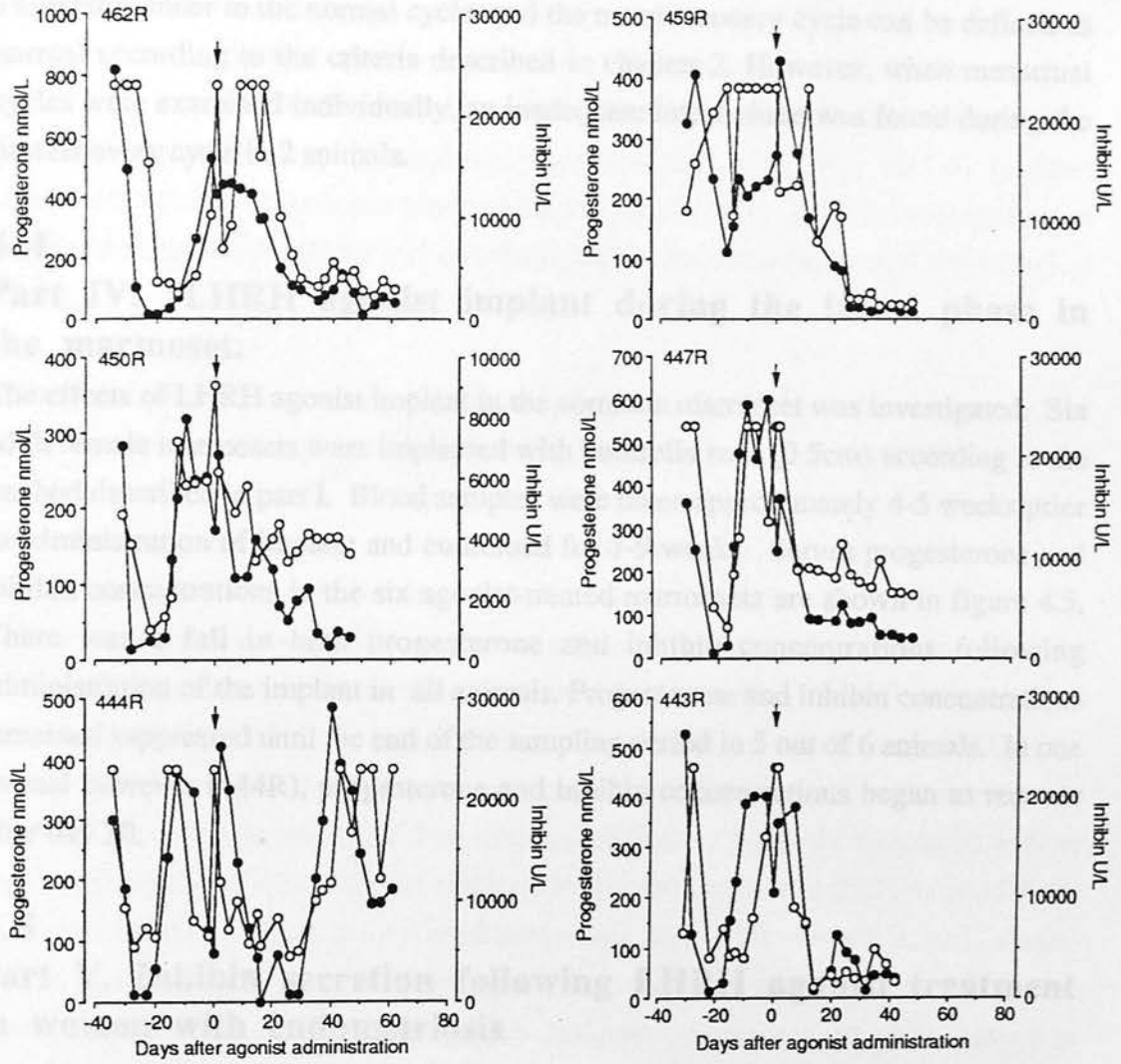


Fig. 4.5. Serum concentrations of progesterone (●) and inhibin (O) in 6 female marmosets following administration of LHRH agonist implant (arrow).

luteal phase implanted animals. Serum progesterone and inhibin concentrations during the recovery cycle in 10 animals is shown in figure 4.4. Inhibin concentrations rose in a similar manner to the normal cycle and the mean recovery cycle can be defined as normal according to the criteria described in chapter 2. However, when menstrual cycles were examined individually, an inadequate luteal phase was found during the first recovery cycle in 2 animals.

4.4
Part IV. LHRH agonist implant during the luteal phase in the marmoset.

The effects of LHRH agonist implant in the common marmoset was investigated. Six adult female marmosets were implanted with buserelin rods (0.5cm) according to the method described in part I. Blood samples were taken approximately 4-5 weeks prior to administration of implant and continued for 7-9 weeks. Serum progesterone and inhibin concentrations in the six agonist-treated marmosets are shown in figure 4.5. There was a fall in both progesterone and inhibin concentrations following administration of the implant in all animals. Progesterone and inhibin concentrations remained suppressed until the end of the sampling period in 5 out of 6 animals. In one animal however (444R), progesterone and inhibin concentrations began to recover after day 30.

4.5
Part V. Inhibin secretion following LHRH agonist treatment in women with endometriosis

In an extensive clinical study designed by Dr. Hamish Fraser, with the clinical collaboration of Dr. R. Haining and Dr. S.K.Smith, the long-term suppression of ovarian function by a luteinizing-hormone releasing-hormone agonist implant was investigated in patients with endometriosis (Fraser et al., 1990). Ten endometriosis patients received luteinizing hormone releasing hormone (LHRH) agonist (buserelin) implant injections (3.3mg (n=4) and 6.6mg (n=6) s.c.). Serum LH and FSH were lowered by day 14. Luteinizing hormone remained at basal concentrations while FSH returned to values in the low-normal range of the menstrual cycle by day 35. At the end of the luteal phase during which treatment commenced, urinary oestrone and pregnanediol declined. Time to first ovulation ranged from 100-194 days (median 118 days) in the 3.3mg group and 79-290 (median 178 days) in the 6.6mg group.

Measurement of inhibin

Serum inhibin concentrations were measured in the samples from the ten subjects from the day of administration of implant until approximately 200 days post-treatment. Serum concentrations of inhibin in two of the subjects in the study are illustrated in figure 4.6 . The profile of inhibin levels in subject Y is representative of 9 out of the 10 subjects. Inhibin concentrations were initially very high due to implant administration during the luteal phase of the menstrual cycle. Serum concentrations of inhibin then declined rapidly and remained suppressed until the end of the sampling period.

Subject X however demonstrated abnormally high levels of inhibin-like activity which remained elevated throughout the entire sampling period. Serum samples from subject X were pooled into pre- and post- implant and serially diluted in the inhibin radioimmunoassay. Both pools were found to be non-parallel to the partially purified human follicular fluid standard in the inhibin assay (fig.4.7). To investigate if this effect was due to a non-specific interaction of products in the subject's serum with the inhibin RIA, plasma and serum from the same blood sample collected post-treatment were assayed and both were found to be non parallel to the standard curve (fig.4.8). It was then investigated if protease enzymes present in the subject's serum were resulting in tracer degradation. Pre- and post-treatment pools were assayed with or without protease inhibitors (20mM ethylenediaminetetraaceticacid (EDTA) and 20mM N-ethylmaleimide (NEM) (initial concentrations), Sigma Chemical Co. Ltd., Poole) and no significant differences in binding of tracer, with or without protease inhibitors, was found (fig.4.9). Furthermore, the pre- and post-treatment pools were assayed in the *in vitro* sheep pituitary cell inhibin bioassay (Tsonis et al., 1986) by Miss Linda Harkness and no bioactivity was present.

4.6 Discussion

The administration of LHRH agonist resulted in a suppression of pituitary-ovarian function in the stumptailed macaque, common marmoset monkey and human. Treatment with LHRH agonist during the follicular and luteal phase in the macaque resulted in a transitory rise in both steroids and inhibin. Thereafter, these hormones were suppressed to follicular phase values. All animals returned to ovulation by 23 weeks after implant. Administration of LHRH agonist in the marmoset resulted in suppression of progesterone and inhibin in all animals. The initial stimulation of steroids and inhibin, noted in the macaque and human, was not observed in the marmoset. However, this may be the result of infrequent sampling. The degree of suppression by LHRH agonist in the marmoset was variable. Inhibin and

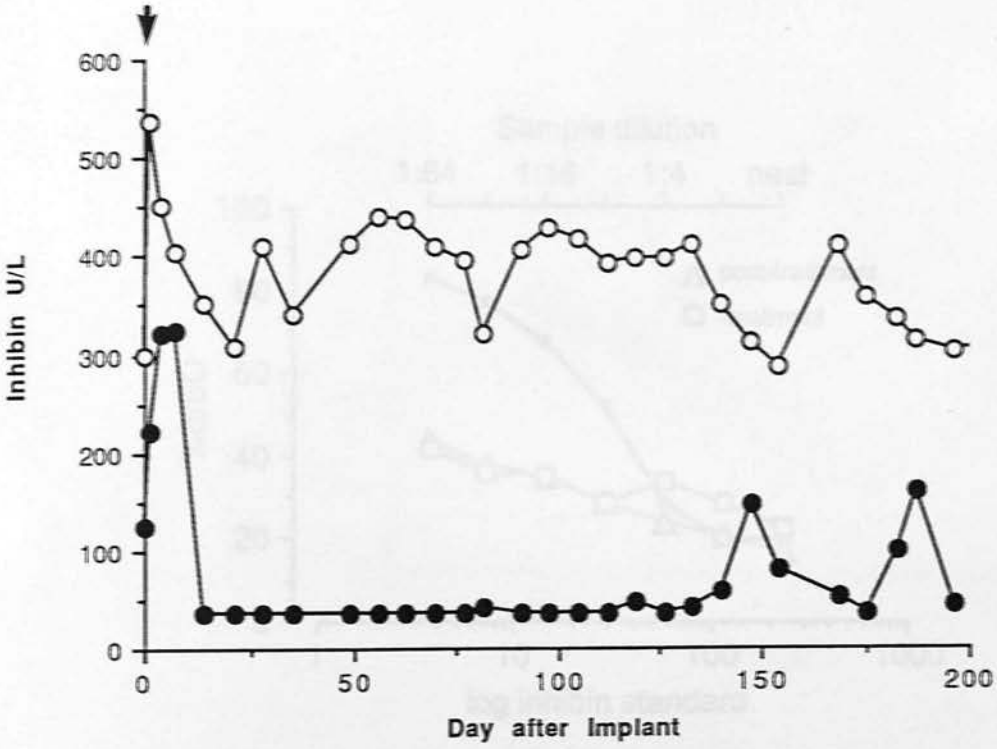


Fig. 4.6. Serum concentrations of inhibin in subject X (○) and subject Y (●) following administration of implant (arrow).

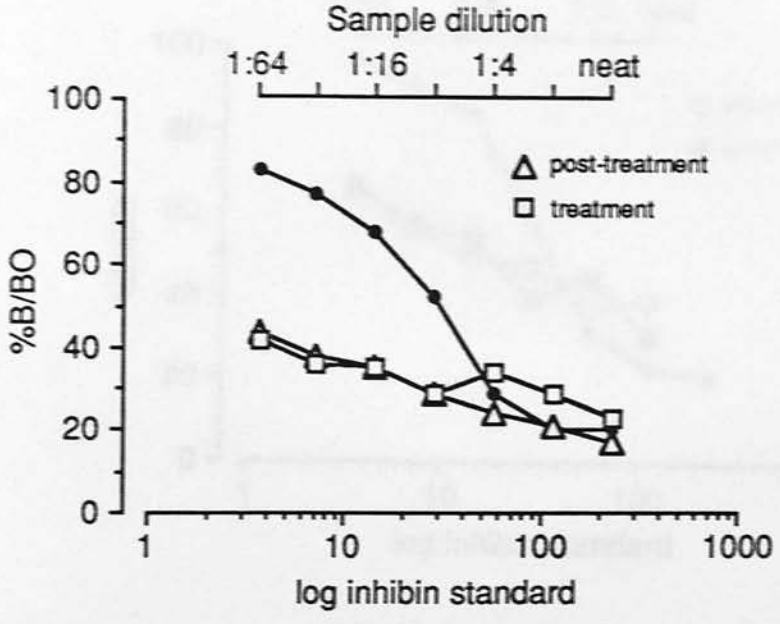


Fig. 4.7. Displacement curves demonstrating non-parallelism between in-house inhibin standard (●) with treatment and post-treatment serum samples from subject X.

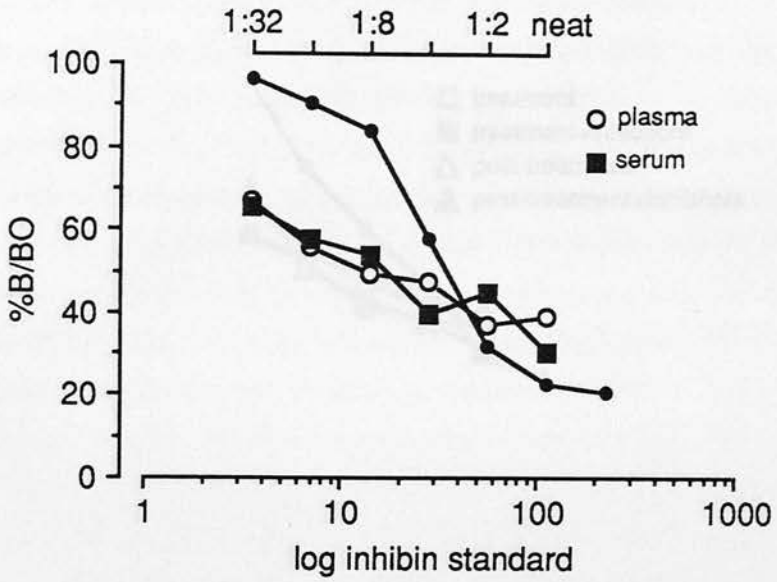


Fig. 4.8. Displacement curves demonstrating non-parallelism between the in-house inhibin standard (●) with plasma and serum samples from subject X.

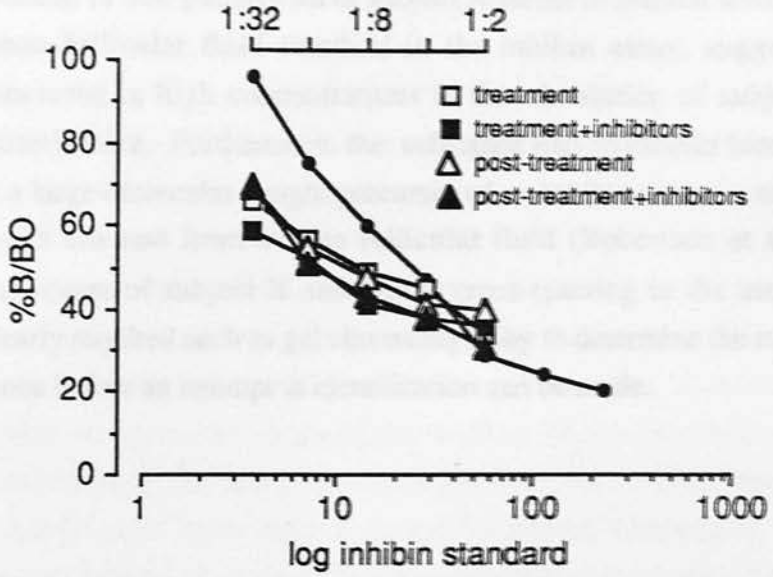


Fig. 4.9. Displacement curves demonstrating non-parallelism between in-house standard (●) with treatment and post-treatment serum samples from subject X.

progesterone were suppressed to below follicular phase levels in 4 out of 6 animals. The recovery period was also variable with one animal achieving normal luteal phase inhibin and progesterone levels by day 30 after agonist administration. Inhibin levels were also suppressed to below follicular phase values following agonist administration in women.

The demonstration of non-parallelism of subject X serum or plasma with the partially purified human follicular fluid standard in the inhibin assay, suggests that the substance measured in high concentrations in the circulation of subject X is not immunoreactive inhibin. Furthermore, the substance had no inhibin bioactivity. It is possible that a large molecular weight precursor of an inhibin subunit, similar to pro-αC and pro-αN isolated from bovine follicular fluid (Robertson et al., 1989), is present in the serum of subject X and this is cross-reacting in the assay. Further studies are clearly required such as gel chromatography to determine the molecular size of this substance before an attempt at identification can be made.

Chapter 5

Immunocytochemical localization of inhibin in the primate ovary.

Part I. Development of immunocytochemical method.

5.1.1 Introduction

The aim of the studies in this chapter was to develop an immunocytochemical method for the cellular localization of inhibin subunits within the primate ovary. The initial intention was to set up the technique of immunocytochemistry in the laboratory using an established method and to adapt this to localise inhibin subunits. The technique for the immunolocalization of oxytocin was chosen as the presence of oxytocin has been well established in a variety of species such as the cow (Guldenaar, 1984; Kruip et al., 1985) and sheep (Watkins, 1983). Oxytocin has also been demonstrated immunocytochemically in the cynomolgus monkey (Khan-Dawood et al., 1983), baboon (Khan-Dawood, 1987a) and human (Khan-Dawood, 1987b). However, these findings in the primate have been disputed by others (Auletta et al., 1988b). Therefore, the localization of oxytocin in the bovine corpus luteum by the peroxidase-anti-peroxidase (PAP) method was set up as a model according to the method of Guldenaar (1984).

This chapter details the development of the immunocytochemical method which was eventually employed to localize inhibin subunits in the primate ovary. The initial PAP method used resulted in specific localization of oxytocin but with a high proportion of non-specific background staining. Experiments were therefore set up to try and improve the amount of background staining by modifying:

(a) tissue pretreatment to reduce:
    (i) endogenous peroxidase activity
    (ii) hydrophobic interactions
    (iii) antigenic masking.
(b) tissue processing
(c) tissue fixation
(d) antibody dilution and incubation time
(e) chromagen

These modifications were then applied to a more sensitive avidin-biotin immunoperoxidase technique which proved to be the most sensitive and produced specific immunostaining with minimum background staining.

5.1.2 Materials and Methods

5.1.2.1 Tissue collection and fixation

Bovine ovaries were collected from the abattoir and corpora lutea dissected out within 2h of removal from the animal. Whole corpora lutea were fixed by immersion using 2.5% glutaraldehyde (v/v) (TAAB Laboratories Equipment Ltd., Aldermaston, Berkshire) and 4.0% paraformaldehyde (w/v) (Aldrich Chemical Company Ltd., Gillingham, Dorset) in 0.1M sodium cacodylate buffer (Agar Scientific Ltd., Stansted, Essex) (fixative 1) at pH 7.4 for 24h.

5.1.2.2 Preparation of tissue sections

After fixation, corpora lutea were frozen and tissue sections (20μm) were cut with a cryostat (2800 Frigocut, Reichert-Jung, Cambridge Instruments, Bar Hill, Cambridge). All frozen sections throughout these studies were cut by Mr. Mike Millar and paraffin sections were prepared by myself and Mike Millar.

5.1.2.3 Peroxidase-anti-peroxidase method

Tissue sections were collected into small glass vials containing Tris-NaCl buffer (0.05M Tris(hydroxymethyl)methylamine (BDH Chemicals Ltd.), 0.9% NaCl, pH 7.6) and washed in Tris-NaCl-Triton-X (Tris-NaCl buffer containing 0.5% Triton-X-100 (Sigma Chemical Company Ltd., Poole, Dorset). The sections were incubated with primary antiserum (rabbit anti-oxytocin antibody R1-5 (Guldenaar, 1984), gift from Dr. I.C.A.F. Robinson, National Institute for Medical Research, Mill Hill) at a dilution of 1:100 for 1h at room temperature followed by overnight incubation at 4°C. Control sections were set up using normal rabbit serum (DAKO Ltd., High Wycombe, Bucks.) at a dilution of 1:100 in place of primary antibody. The sections were then immunostained using a peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979). Following a wash in Tris-NaCl containing 2% normal swine serum (DAKO Ltd.), sections were incubated with secondary antibody (swine-anti-rabbit) (DAKO Ltd.) at a dilution of 1:30 for 30 min. Sections were washed again for 15 min in Tris-NaCl containing 2% normal swine serum (DAKO Ltd.). Rabbit peroxidase anti - peroxidase complex (PAP complex) (DAKO Ltd.) at a dilution of 1: 100 was then applied for 30 min. Sections were then washed for 15 min in Tris - NaCl. The product was then visualized using 0.5 mg 3,3'-diaminobenzidine

tetrahydrochloride/ml (DAB) (Sigma Chemical Company Ltd.) in Tris-NaCl containing 0.1ml 3% hydrogen peroxide (BDH Chemicals Ltd.). A few drops of the DAB solution were applied to the tissue sections for 3-5 min. Following a wash in Tris-NaCl, the sections were counterstained in haemotoxylin (BDH Chemicals Ltd.), dehydrated and mounted using Histomount (National Diagnostics, Highland Park, New Jersey, U.S.A.) for microscopic examination.

5.1.3 Results

PAP method

Specific staining corresponding to oxytocin in three bovine corpora lutea with absence of staining in the control sections was obtained using this protocol in three separate immunocytochemical experiments. However, there was excessive background staining in both treated and control tissue sections. The experimental protocol therefore had to be modified in order to achieve the optimum conditions for specific immunostaining with the minimum background staining.

5.1.4 Modifications to immunostaining protocol

Problems: causes and solutions

5.1.4.1 Background staining: Tissue pre-treatment

(a) Endogenous peroxidase activity

Endogenous peroxidase activity is a major cause of non-specific background staining. Peroxidase activity results in the decomposition of H_2O_2 and is a common property of all haemoproteins such as haemoglobin. The highly vascularised structure of the corpus luteum suggests that this tissue may contain a lot of endogenous peroxidase activity. The most frequently used procedure for the suppression of endogenous peroxidase activity is the incubation of sections with hydrogen peroxide in methanol. Therefore in an attempt to reduce background staining due to endogenous peroxidase activity, sections were incubated with 3% (v/v) hydrogen peroxide (BDH Chemicals Ltd.) in methanol (BDH Chemicals Ltd.) for 30 min. This resulted in a decrease in the amount of background staining in tissue sections from three bovine corpora lutea in two separate immunocytochemical experiments.

(b) Hydrophobic interactions

Another major cause of backgound is due to the hydrophobic binding between tissue proteins and the antibody molecules. The most widely practiced measure to reduce background due to hydrophobic interaction is the use of an incubation step with a

blocking protein either separately or added to the diluent, prior to application of primary antibody. Tissue sections were therefore incubated for 30 min with a normal non-immune swine serum (DAKO Ltd.) (at a dilution of 1: 5 with Tris-NaCl buffer). This treatment was found to reduce background following the hydrogen peroxide treatment in tissue sections from three bovine corpora lutea tested in three immunocytochemical experiments. Furthermore, in the previous experiments, the addition of 5% bovine serum albumin (Sigma Chemical Co.Ltd.) to the blocking serum together with antibody diluents consisting of 1:5 normal swine serum/Tris-NaCl further reduced background staining. The combination of all of these pre-treatments further reduced the background staining and these pre-treatments were all adopted in the immunocytochemical protocol. Examples of results obtained are shown in plate 5.1 (A and B). 5.1A shows specific staining representing oxytocin in the large luteal cells of the bovine corpus luteum. No specific staining was present in 5.1B where primary antibody was substituted by normal rabbit serum. However, a high degree of non-specific background staining was present in both control and treated tissue and hence further modifications to the protocol were necessary.

(c) Antigenic masking

A further source of background staining may be the result of tissue fixation. Tissue proteins are rendered more hydrophobic by fixation with aldehyde-containing reagents such as formalin and glutaraldehyde. The increased hydrophobicity is often the result of cross-linking of reactive epsilon- and alpha-amino acids, both within and between adjacent protein molecules, which mask antigenic binding sites. An intermediate step between hydrogen peroxide/methanol and blocking serum treatments of incubation of tissue sections in 0.1% trypsin (BDH Chemicals Ltd.) in 0.1% calcium chloride (BDH Chemicals Ltd.) at 37°C was therefore included. This treatment was tested to determine if it would 'unmask' any antigenic sites masked by the fixative. However, the trypsin treatment was included in four immunocytochemical experiments and had no effect in reducing background. Trypsin pretreatment was therefore not included in the protocol.

5.1.4.2 Tissue Processing

The above experiments were carried out on tissue sections of 20μm thickness. The immunostaining obtained with tissue of varying thickness was then compared and also the results obtained between frozen and paraffin-embedded tissue sections. Three bovine corpora lutea were frozen and tissue sections cut at 3μm and 7μm in a cryostat, fixed in acetone (BDH Chemicals Ltd.) for 15min and stained for the presence of oxytocin as described previously. Specific staining for oxytocin is shown in plate

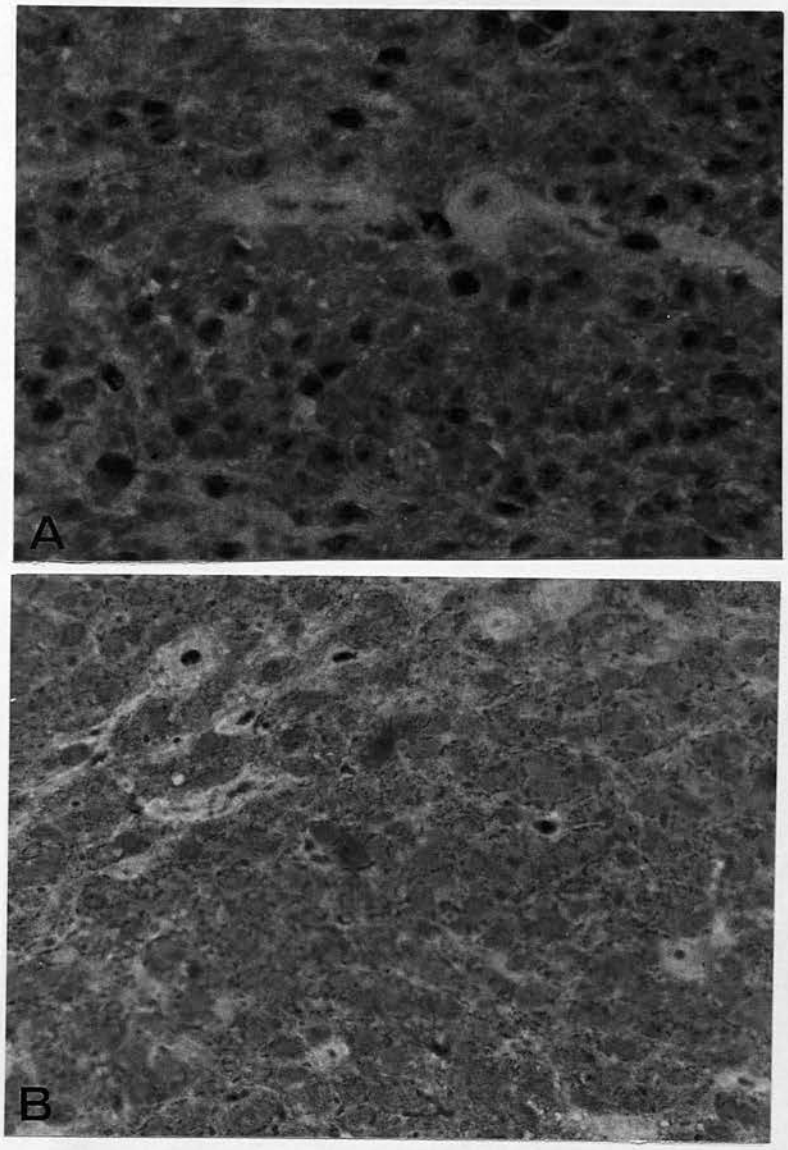


Plate 5.1. Localization of oxytocin in 20μm sections of bovine corpus luteum (A) using PAP method. In section B, primary antiserum was substituted by normal rabbit serum. High degree of background staining is present. (x 25).

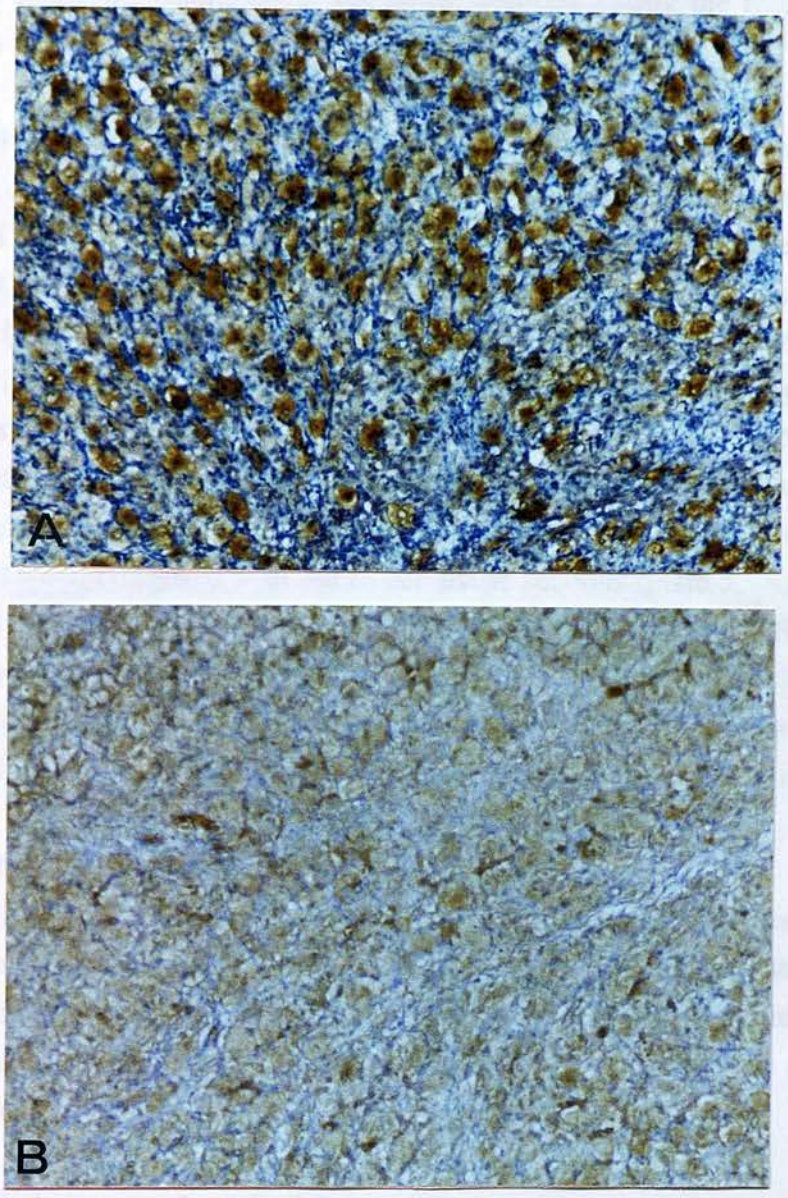


Plate 5.2. Localization of oxytocin in 7μm frozen sections of bovine corpus luteum using PAP method. In section B, primary antiserum was substituted by normal rabbit serum. High degree of tissue damage and background staining is present. (x 25).

5.2A. Absence of staining is noted in 5.2B where normal rabbit serum was used as a control. However, although specific staining was obtained, there was substantial background staining and poor tissue morphology was maintained. This was most likely due to crystal formation in the tissue sections during the freezing process resulting in tissue damage.

The results obtained with frozen tissue were then compared with fixed, paraffin-embedded sections. Following fixation in fixative 1, three corpora lutea were dehydrated in a series of alcohols (70%, 80%, 90%, 95%, absolute), Histoclear (National Diagnostics, Highland Park, New Jersey, U.S.A.) and wax in a tissue processor (model 2LE, Shandon, Runcorn, Cheshire). The tissues were then embedded in paraffin wax in a tissue embedding centre (Reichert Jung, Cambridge Instruments). 4µm sections were then cut with a microtome (Supercut 2050, Reichert Jung, Cambridge Instruments). By applying the PAP method to these sections, specific immunostaining representing oxytocin was obtained with decreased background when compared with the frozen tissue sections (plate 5.3A) and absence of staining was noted in the control section (5.3B). 4µm, paraffin embedded sections were therefore used in preference to frozen sections in the immunocytochemical staining method. However, it was considered that the level of background staining could be reduced so that further modifications were investigated.

5.1.4.3 Tissue fixation

The ideal fixative for immunoperoxidase studies should give good morphological preservation without destroying the immunoreactivity of the antigen. It should prevent extraction, diffusion and displacement of the antigen and not interfere with subsequent antigen/antibody reactions. The effect of using different fixatives on the resultant immunostaining was therefore investigated.

Three corpora lutea were fixed for 24h in either:

(i) fixative 1

(ii) 4% paraformaldehyde

(ii) Bouin's fixative (75ml of saturated (1.2% w/v) of aqueous picric acid, 25ml formalin (40% w/v formaldehyde) and 5ml glacial acetic acid, all purchased from BDH Chemicals Ltd.).

Tissues were then processed as described above for paraffin sections. 4µm tissue sections were then immunostained for oxytocin using the PAP method. The most

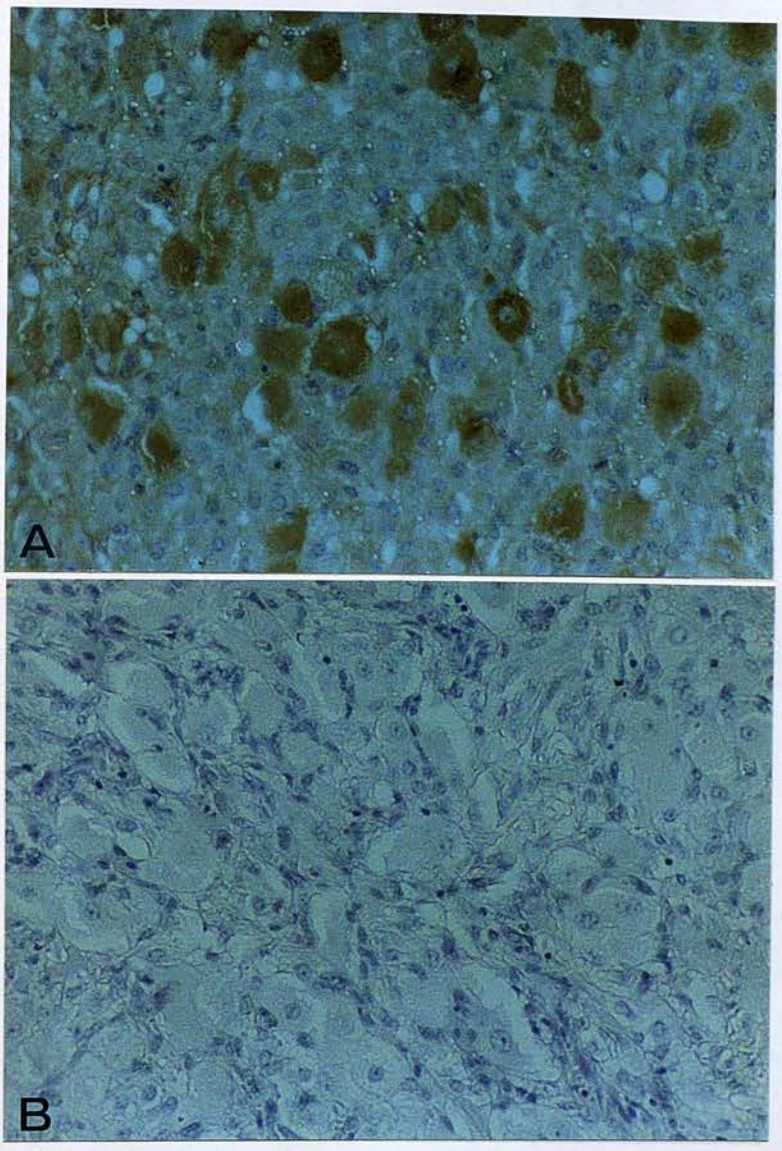


Plate 5.3. Localization of oxytocin in 4μm paraffin sections of bovine corpus luteum using PAP method. In section B, primary antiserum was substituted by normal rabbit serum. Background staining is reduced. (x 50).

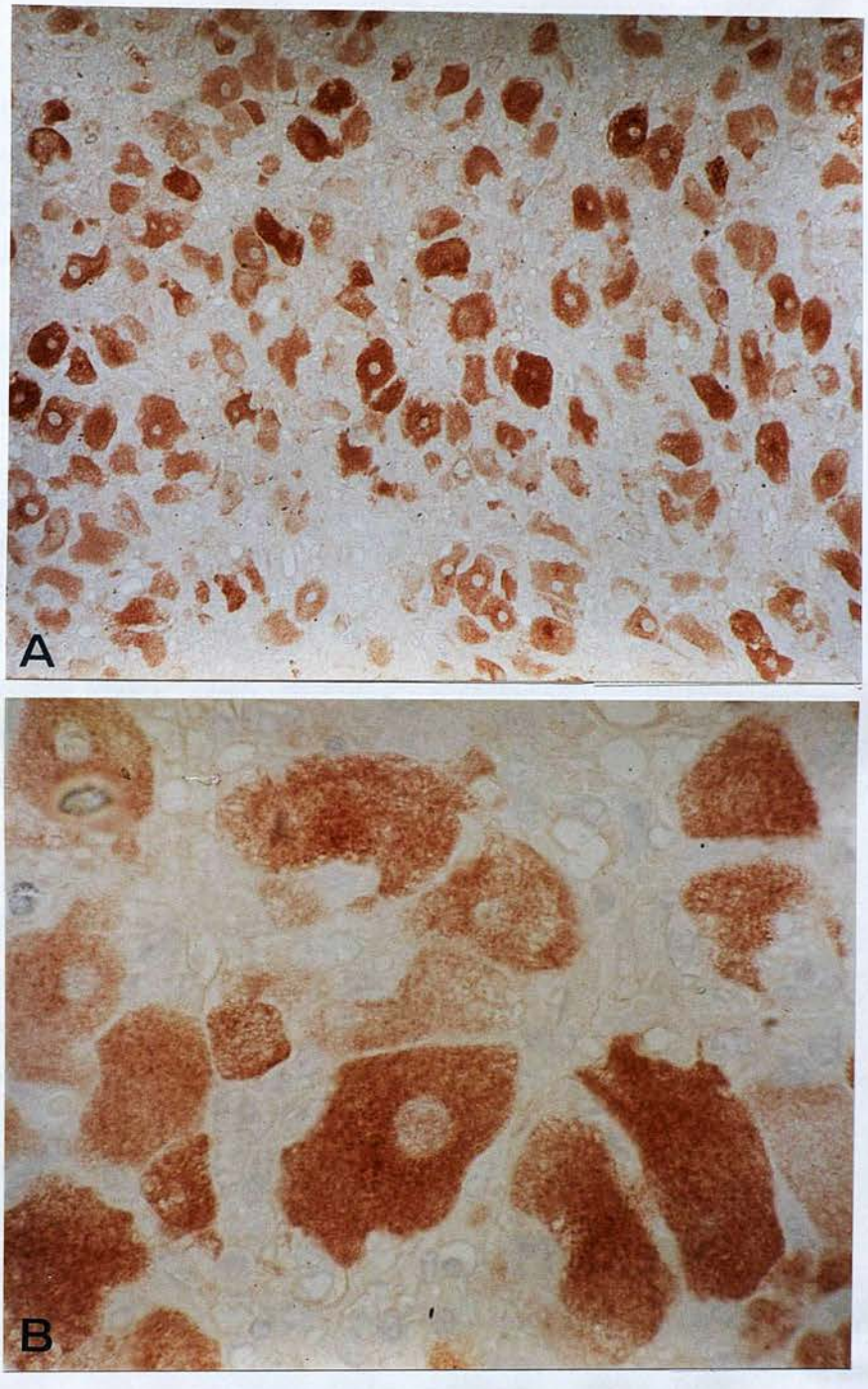


Plate 5.4. Localization of oxytocin in 4μm paraffin sections of bovine corpus luteum using avidin-biotin method shown in low power (A, x 40) and high power (B, x 400).

specific staining with decreased background was achieved using fixative 1 (plates 5.3 and 5.4). Thus fixative 1 was used routinely in the immunocytochemical method.

5.1.4.4 Antibody

The optimum antibody titre is defined as the highest dilution of an antiserum which results in optimal specific staining with the least amount of background staining. The antibody (R1-5) was used at dilutions of 1:100-1:10000 incubating from 30 min at room temperature to overnight at 4°C in tissue sections from four different corpora lutea. 1:400 was found to be the optimum dilution when incubated for 1h at room temperature (plate 5.4) and this procedure was adopted in the immunocytochemical protocol.

5.1.4.5 Chromagens

The key enzyme involved in the immunocytochemical reaction in this chapter is horseradish peroxidase. Peroxidase activity in the presence of an electron donor results in the formation of an enzyme-substrate complex followed by the oxidation of the electron donor. There are several electron donors which, upon being oxidised, become coloured products and are therefore called chromagens. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density. DAB was found to be an extremely effective chromagen for localizing oxytocin (plate 5.3). However, in an experiment to investigate the enhancing properties of osmium tetroxide, sections of two corpora lutea were immunostained for oxytocin using DAB and visualized with and without osmium tetroxide exposure. Exposure to osmium tetroxide resulted in increased background staining and DAB was therefore used in the immunocytochemical protocol without osmium tetroxide exposure.

3-Amino-9-ethylcarbazole (AEC) produces a red end-product upon oxidation which is alcohol soluble. Specimens processed with AEC must therefore not be immersed in alcohol or alcoholic solutions. Instead, an aqueous counterstain and mounting medium must be used. AEC is also susceptible to further oxidation and when exposed to excessive light, will fade in intensity. Tissue sections treated with AEC must therefore be stored in the dark. In an experiment to investigate the visualizing properties of AEC, two corpora lutea were immunostained for oxytocin using either AEC or DAB as a chromagen. AEC was found to produce more intense

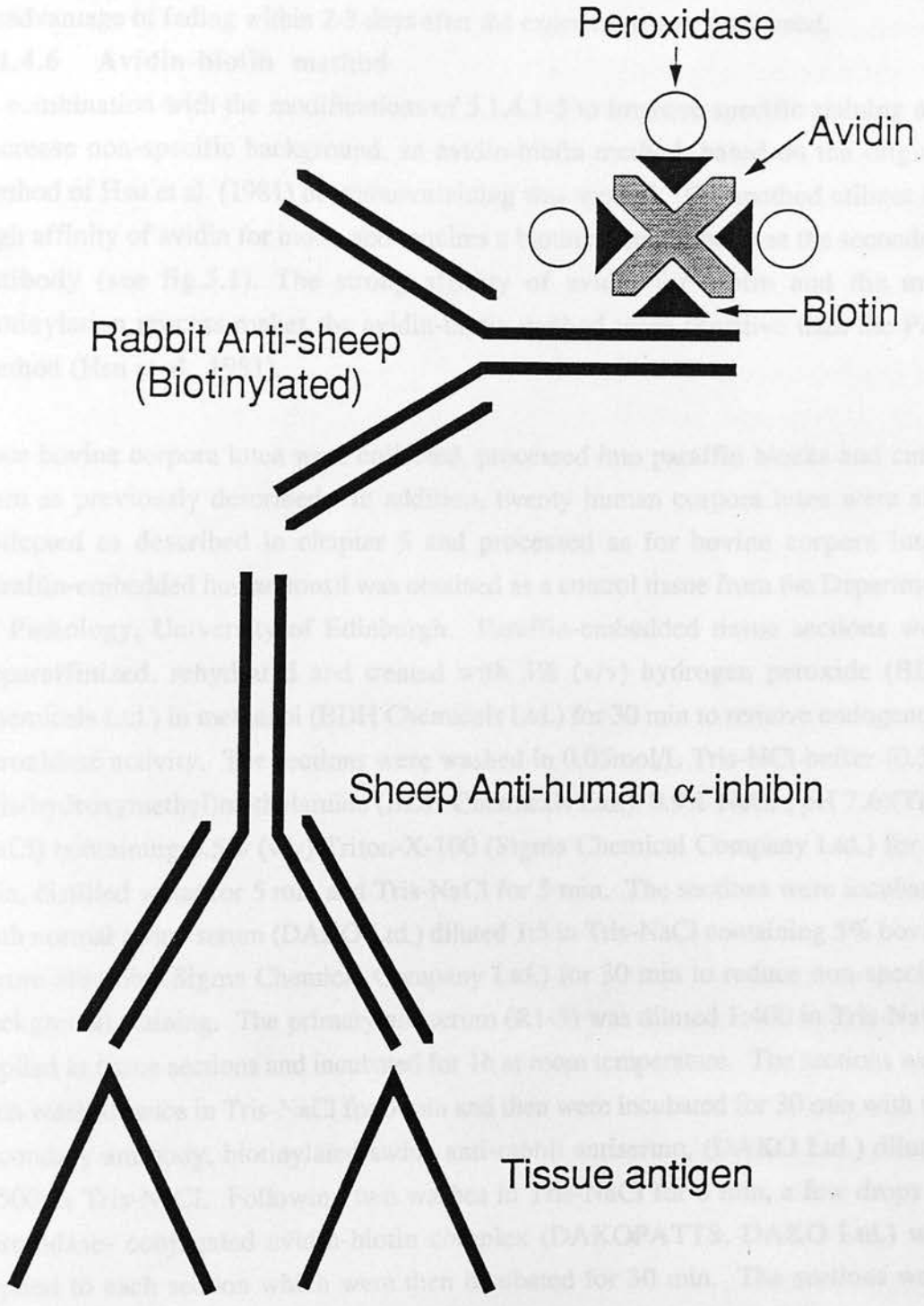


Fig. 5.1. Schematic diagram of avidin-biotin technique.

immunostaining (plate 5.4) when compared with DAB although it had the disadvantage of fading within 2-3 days after the experiment was performed.

5.1.4.6 Avidin-biotin method

In combination with the modifications of 5.1.4.1-5 to improve specific staining and decrease non-specific background, an avidin-biotin method, based on the original method of Hsu et al. (1981) of immunostaining was applied. This method utilizes the high affinity of avidin for biotin and requires a biotinylated antibody as the secondary antibody (see fig.5.1). The strong affinity of avidin for biotin and the mild biotinylation process makes the avidin-biotin method more sensitive than the PAP method (Hsu et al., 1981).

Four bovine corpora lutea were collected, processed into paraffin blocks and cut at 4μm as previously described. In addition, twenty human corpora lutea were also collected as described in chapter 3 and processed as for bovine corpora lutea. Paraffin-embedded human tonsil was obtained as a control tissue from the Department of Pathology, University of Edinburgh. Paraffin-embedded tissue sections were deparaffinized, rehydrated and treated with 3% (v/v) hydrogen peroxide (BDH Chemicals Ltd.) in methanol (BDH Chemicals Ltd.) for 30 min to remove endogenous peroxidase activity. The sections were washed in 0.05mol/L Tris-HCl buffer (0.5M Tris(hydroxymethyl)methylamine (BDH Chemicals Ltd.), 0.9% NaCl , pH 7.6)(Tris-NaCl) containing 0.5% (v/v) Triton-X-100 (Sigma Chemical Company Ltd.) for 10 min, distilled water for 5 min and Tris-NaCl for 5 min. The sections were incubated with normal swine serum (DAKO Ltd.) diluted 1:5 in Tris-NaCl containing 5% bovine serum albumin (Sigma Chemical Company Ltd.) for 30 min to reduce non-specific background staining. The primary antiserum (R1-5) was diluted 1:400 in Tris-NaCl, applied to tissue sections and incubated for 1h at room temperature. The sections were then washed twice in Tris-NaCl for 5 min and then were incubated for 30 min with the secondary antibody, biotinylated swine anti-rabbit antiserum, (DAKO Ltd.) diluted 1:500 in Tris-NaCl. Following two washes in Tris-NaCl for 5 min, a few drops of peroxidase- conjugated avidin-biotin complex (DAKOPATTS, DAKO Ltd.) was applied to each section which were then incubated for 30 min. The sections were again washed twice in Tris-NaCl for 5 min. The peroxidase activity was then visualized using 0.5mg 3,3'-diaminobenzidine tetrahydrochloride / ml (DAB)(Sigma Chemical Company Ltd.) in Tris-NaCl containing 0.1ml of 3% hydrogen peroxide (BDH Chemicals Ltd.) which was applied to the sections for 3-5 min. Alternatively, 3-amino-9-ethyl carbazole (AEC) (Sigma Chemical Company Ltd.) was applied as a chromagen. 4 mg of 3 - amino - 9 - ethyl carbazole was dissolved in 1ml of

N,N-dimethylformamide (BDH Chemicals Ltd.). 14ml of acetate buffer, pH 5.2, (prepared from 210ml of 0.1N acetic acid and 790ml of 0.1M sodium acetate, both from BDH Chemicals Ltd.) and 0.15ml of 3% hydrogen peroxide then added. The mixture was then filtered, applied to the tissue and incubated for 5-15min at room temperature. Following a wash in Tris-NaCl, the sections were counterstained in haemotoxylin (BDH Chemicals Ltd.), dehydrated and mounted using Histomount (National Diagnostics) for DAB-treated sections or Aquamount (National Diagnostics) for AEC-treated sections and examined microscopically.

To test the specificity of staining, control sections were set up with antiserum which had been preabsorbed overnight with 10μM oxytocin (Cambridge Research Biochemicals Ltd., Harston, Cambridge) in place of primary antiserum. Control sections were also set up substituting primary antiserum with normal rabbit serum at 1:400.

5.1.5 Results

Avidin-biotin method

The results from this method are shown in plate 5.4A and 5.4B. The avidin-biotin system demonstrated specific staining in the large luteal cells of the bovine corpus luteum with minimum background staining as shown in low power (5.4A) and high power (5.4B). Less background staining was obtained using this method in comparison with the PAP method (plate.5.3). No immunostaining was present in the tissue sections where primary antiserum was preabsorbed with oxytocin or when normal swine serum was used in place of primary antibody. No immunostaining was present in the sections of human tonsil (plate. 5.7A). The optimum staining protocol had therefore been obtained. No immunostaining was detected in tissue sections from twenty different human corpora lutea from various stages of the luteal phase which were examined for the presence of oxytocin in ten immunocytochemical experiments.

5.1.6 Discussion- part I.

The finalized protocol for the immunocytochemical method is shown in figure 5.2. The absence of staining in control tissue sections and in human tonsil demonstrated antibody specificity. The finding of oxytocin in the large luteal cells of the bovine corpus luteum is in agreement with the findings of Guldenaar et al. (1984) and Kruip et al. (1985).

Treatment	Time
3% hydrogen peroxide in methanol	30 min
Tris-NaCl/Triton-X wash	10 min
Distilled water	5 min
Tris-NaCl	5 min
Blocking serum	30 min
Primary antiserum	30 min
Tris-NaCl wash	2 x 5min
Secondary antiserum	30 min
Tris-NaCl wash	2 x 5 min
AB complex	30 min
Tris-NaCl wash	2 x 5 min
DAB/AEC	3 - 5 min
Tris-NaCl wash	5 min
Counterstain	

Fig. 5.2 Final protocol for immunostaining method

The absence of staining in any of the human corpora lutea is in agreement with the findings of Auletta et al. (1988b) but in contrast with those of Khan-Dawood (1987b). However, the presence of oxytocin in the human corpus luteum is a controversial issue which has still to be resolved (see chapter 1).

Part II Immunocytochemical localization of inhibin in the primate ovary

5.2.1 Introduction

At the time when these studies were carried out, there were no reports of immunocytochemical localization of inhibin in the primate corpus luteum. Furthermore, there were no reports on the localization of inhibin within a specific cell type in the ovary. The aim of the following study was therefore to determine, firstly, whether inhibin α-subunit could be detected immunocytochemically within the human corpus luteum, human follicle and in pre-ovulatory follicles and corpora lutea from the stumptailed macaque; secondly, whether inhibin α-subunit is localized in a specific cell type in the primate corpus luteum and whether the intensity or distribution of inhibin α-subunit immunostaining in the human corpus luteum varies throughout the different stages of the luteal phase and finally, in a preliminary study, to determine if inhibin β_A-subunit could be detected immunocytochemically within the human corpus luteum and if so, was inhibin β_A-subunit localized to a specific cell type.

5.2.2 Materials and Methods

5.2.2.1 Tissue Preparation

(i) Human tissue

Human corpora lutea at early (1-4 days since ovulation), mid (5-9 days since ovulation) and late (10-14 days since ovulation) stages of the luteal phase (n=4 per group) were obtained from patients undergoing hysterectomy by Dr. Peter Illingworth. The stage of the menstrual cycle was determined as described in chapter 2. Informed consent was obtained from all subjects and ethical approval for the study was obtained from the Lothian Area Ethical Committee, Reproductive Medicine Subcommittee, Edinburgh.

Human corpora lutea were collected into ice-cold phosphate-buffered saline (ICN Flow Ltd., Rickmansworth, Herts., U.K.) and blood clots and excess connective tissue removed as described in chapter 3. Five human follicles were also collected in a

similar manner. The tissues were fixed for 24 h in fixative 1, dehydrated, embedded in paraffin wax and cut into 4μm serial sections as described in part 1.

(ii) Macaque tissue

Four macaque ovaries were collected by Dr. Hamish Fraser and immersed immediately into liquid nitrogen (one ovary from no. 66 and one ovary from 83) or into fixative 1(for 24h) (two ovaries from no.78). 4μm sections were then cut using a cryostat/microtome. The frozen sections were then fixed in a buffered picric acid-formaldehyde solution (a modification of Bouin's fluid) for 10min at 4°C prepared as follows:

20g of paraformaldehyde was added to 150ml of a double-filtered, saturated aqueous solution of picric acid. The solution was then heated and cleared with a few drops of concentrated sodium hydroxide. The solution was then filtered, cooled and made up to 1000mls with phosphate buffer (prepared with 3.31g/L of $NaH_2PO_4.H_2O$ and 33.77g/L of $Na_2HPO_4.7H_20$). Following fixation, tissue sections were washed in phosphate buffered-saline pH 7.4 (prepared from 9g/L NaCl, 3.5g/L Na_2HPO_4 and 11.88g/L $Na_2PO_42H_20$) for 30min at 4°C. All chemicals were purchased from BDH Chemicals Ltd.

The stage of the menstrual cycle was determined by radioimmunoassay for progesterone, oestradiol and FSH from blood samples taken daily (as described in chapter 2) for two weeks prior to removal of the ovaries. Radioimmunoassays used as described in chapters 2-4.

5.2.2.2 Antiserum

Several antibodies, raised by Dr. Alan McNeilly, were initially tested for the ability to immunolocalize inhibin α-subunit in the human corpus luteum. Three antisera (R146, R147 and R150) were raised in rabbits against the first 1-26 amino acids of the N-terminus of the porcine α-subunit conjugated to ovalbumin. These antisera were titrated at dilutions of 1:100-1:10 000 for various incubation times (30 or 60min at room temperature or overnight at 4°C) using the avidin-biotin method described in part I. However, no immunostaining was obtained using these antisera, at these dilutions or incubation periods, in six human corpora lutea from different stages of the luteal phase.

Two antisera (Y29 and Y33) were raised in sheep against the first 1-23 amino acid sequence of the N-terminus of the human α-subunit (Cambridge Research Biochemicals) conjugated to rabbit gamma globulin (Y29) or porcine thyroglobulin

(Y33) by means of carbodiimide (1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide hydrochloride; Sigma Chemical Co. Ltd.). These antisera were used to establish a radioimmunoassay for the detection of human α-subunit (A.S.McNeilly and W.J.Crow, unpublished).

5.2.2.3 Inhibin α-subunit radioimmunoassay

(i) Assay buffer- 1% bovine serum albumin (BSA)/ phosphate buffered saline (PBS) prepared from:

0.5M phosphate buffer stock consisting of:

133.5g $NaHPO_4.2H_20$ /1.5L

39g $NaH_2PO_4.2H_20$/0.5L

Chemicals from BDH Chemicals Ltd.

Buffer preparation:

300mls of 0.5M stock + 1700ml distilled water

8.76g/L NaCl (BDH Chemicals Ltd.)

0.2g/L thiomersal (Sigma Chemical Co.Ltd.)

20g/L bovine serum albumin (Sigma Chemical Co.Ltd.)

(ii) Assay set-up

Assays included duplicate estimations of total binding, non-specific binding and standards/samples. The following additions were made to each assay tube as described in chapter 2.

100µl standard (Peninsula human 1-23α inhibin standards, range 1-15600 pg/0.1ml, Peninsula Laboratories Inc., Belmont, CA., U.S.A.)

200µl assay buffer

100µl antibody (Y29; 1:2000 or Y33; 1:6000) conjugated to fluorescein isothiocyanate (FITC) by Serono Laboratories, Woking, Surrey.

100µl tracer (^{125}I-Peninsula human 1-23α inhibin, 15 000 counts per tube, iodinated by Miss W.J.Crow using the chloramine T method).

Assays then incubated for 1h at 37°C.

(iii) Assay separation

The immunoreactive - bound iodinated tracer was separated using anti-FITC magnetic particle separation (Serono Laboratories, Woking, Surrey). 200µl of anti-FITC magnetic particles were added to all tubes except the total counts. All tubes were mixed gently and allowed to stand for 5 min. Assay tubes were then placed

onto a magnetic separator (Serono Laboratories) for 2 min, decanted and blotted. 500µl of 0.9% saline/0.2% triton X-100 was then added to all tubes except total counts. The separating and decanting procedure was then repeated and tubes counted in a gamma counter as described in chapter 2. The antisera gave 30% binding of ^{125}I-labelled 1-23α- human inhibin at an initial dilution of 1:2000 (Y29) and 1:6000 (Y33) and parallel displacement curves were obtained with the human 1-23 inhibin standard. Furthermore, parallel displacement curves were obtained with human, ovine and bovine follicular fluid using both Y33 and Y29 (fig.5.3A and 5.3B). In addition, parallel displacements curves were also obtained with dilutions (1:2-1:128) of an extract of a mid-luteal phase human corpus luteum (fig.5.4), prepared by homogenizing 1g tissue/10ml assay buffer. The homogenate was assayed in the presence of two protease inhibitors to prevent tracer degradation: ethylenediaminetetra acetic acid (EDTA) and N-ethyl malaeimide (NEM) both at an final concentration of 2mM in the assay. These protease inhibitors had no effect on the assay as no significant differences were obtained in standard curves with and without protease inhibitors.

5.2.2.4 Immunocytochemistry

The avidin-biotin immunoperoxidase technique, developed in part I was used to localize human inhibin α-subunit and β_A-subunit in the primate ovary with the following modifications:

A. Inhibin α-subunit

(i) Normal rabbit serum (DAKO Ltd.) diluted 1:5 in Tris-NaCl containing 5% bovine serum albumin for 30 min was applied prior to primary antibody to reduce non-specific background staining.

(ii) In a preliminary study to obtain optimum antibody dilution and incubation times, six human corpora lutea were immunostained for inhibin α-subunit using Y29 or Y33 diluted 1:100-1:10 000 in Tris-NaCl containing 1:5 diluted normal rabbit serum and incubated for 30min or 1h at room temperature or 24h at 4°C. Less background staining was obtained with Y33 in comparison with Y29, presumably due to the conjugate used in raising Y29 (rabbit gamma globulin) interfering with the immunostaining system. Y33 was therefore the preferred antibody to use in the staining protocol.

(iii) secondary antibody, biotinylated rabbit anti-sheep, (Vector Laboratories, Peterborough, Cambs., U.K.) diluted 1:500 in Tris-NaCl containing 1:5 diluted normal rabbit serum.

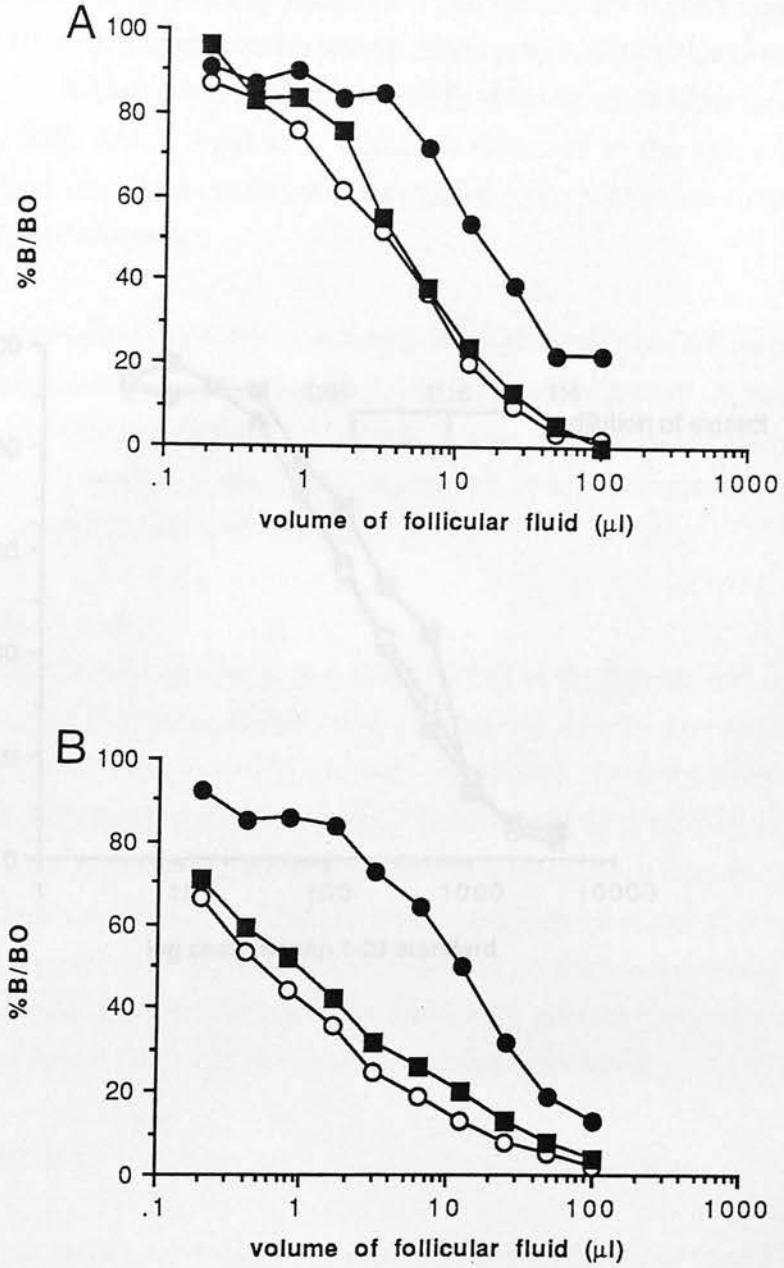


Fig. 5.3. Parallelism was obtained using antisera Y33 (A) and Y29 (B) with human follicular fluid (●), ovine follicular fluid (O) and bovine follicular fluid (■) in the inhibin α-subunit RIA.

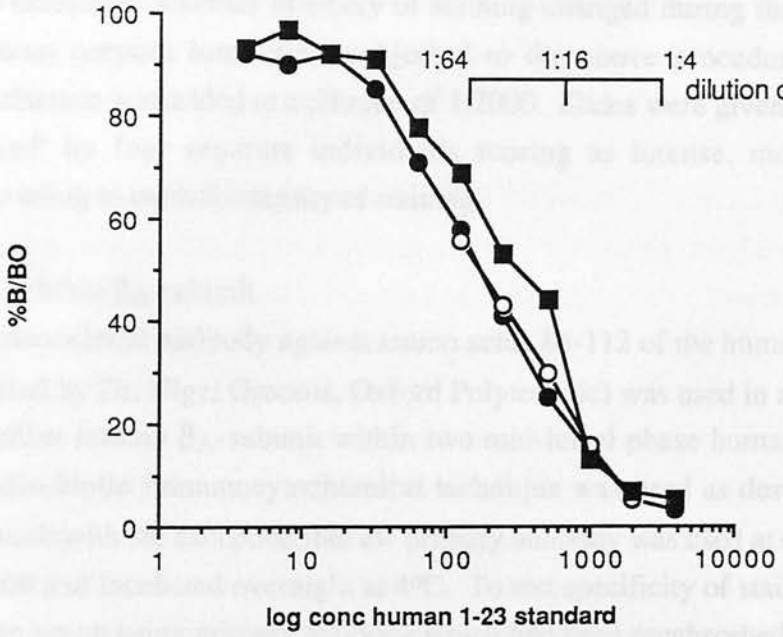


Fig. 5.4. Parallelism was obtained using antisera Y33 (■) and Y29 (●) with extracts of human corpus luteum(O) in the inhibin α-subunit RIA.

To test the specificity of the staining, control sections were set up with primary antiserum which had been preabsorbed overnight with 10μg human 1-23 α inhibin immunogen in place of primary antiserum. Control tissue sections were also set up substituting primary antiserum with normal sheep serum (DAKO Ltd.) at 1:2000. The specificity was further substantiated by serially diluting the primary antiserum from 1:250 to 1:2000, which resulted in a gradual decrease in the overall intensity of staining. Human tonsil was included as a negative control tissue and rat antral follicles as a positive control tissue.

To determine whether intensity of staining changed during the luteal phase, the 12 human corpora lutea were subjected to the above procedure in a single assay. Antiserum was added at a dilution of 1:2000. Slides were given a code and examined 'blind' by four separate individuals scoring as intense, moderate, weak or nil according to overall intensity of staining.

B. Inhibin β_A-subunit

A monoclonal antibody against amino acids 84-112 of the human inhibin β_A-subunit (gifted by Dr. Nigel Groome, Oxford Polytechnic) was used in a preliminary study to localize inhibin β_A-subunit within two mid-luteal phase human corpora lutea. The avidin-biotin immunocytochemical technique was used as described for inhibin α-subunit with the exception that the primary antibody was used at dilutions of 1:100 and 1:200 and incubated overnight at 4°C. To test specificity of staining, control sections were set up using primary antibody which had been preabsorbed overnight with 10μg inhibin β_A peptide. Furthermore, tissue sections were also set up using a non-immune normal mouse serum (DAKO Ltd.) in place of primary antibody.

5.2.3 Results

Intense immunostaining corresponding to inhibin α-subunit was obtained in all human corpora lutea studied (eg. Plate 5.5A). The staining was confined to the granulosa-lutein cells (GL) of the human corpus luteum and varied in intensity within this cell population (Plate 5.6). Absence of staining was obtained within the intrusion of theca-lutein cells (TL) and in surrounding ovarian stromal tissue. No differences were observed between age of the corpus luteum and intensity or distribution of staining and no significant correlation was obtained between 'score' and age of corpus luteum.

Preabsorption of the antiserum with human 1-23 α inhibin immunogen and substitution of antiserum with normal rabbit serum abolished immunostaining (Plate 5.5B), demonstrating specificity of the antibody. No immunostaining was present in sections of human tonsil (5.7A). Positive immunostaining was obtained within granulosa cells of follicles of a premature rat ovary (day 21) (5.7B).

Moderate immunostaining corresponding to inhibin β_A-subunit was obtained in both human corpora lutea studied (Plate 5.8). Furthermore, the staining was also confined to the granulosa-lutein cells within the tissue (Plate 5.9A) and no staining was noted within the theca-lutein cells. Preabsorption of the antibody with human β_A-inhibin immunogen (not shown) and substitution of antibody with normal mouse serum (Plate 5.9B) abolished immunostaining.

It was also attempted to localize inhibin α-subunit in human antral follicles. Positive staining was observed in the granulosa cells (G) of one small follicle (4mm diameter) with absence of staining within the theca cell layers (T) (Plate 5.10). No staining was seen in the four other follicles studied.

Intense inhibin α-subunit immunostaining was observed in the granulosa cells of a large preovulatory macaque follicle no.66 (Plate 5.11). This ovary was obtained on day 12 of the follicular phase on the day of the LH/FSH surge, when oestradiol concentrations were rising and progesterone levels were basal (fig.5.5). No staining was observed within the thecal cell layer, although differentiation between cell populations was more difficult to distinguish in these frozen tissue sections (Plate 5.12A, 5.12B). Moderate immunostaining was observed in a second macaque ovary containing a corpus luteum (no.83) (Plate 5.13). This ovary was removed on days 2-3 of the luteal phase when peripheral progesterone and oestradiol concentrations were rising (fig.5.6). Unfortunately, the cellular morphology of the corpus luteum in frozen tissue sections was poor and cell types could not be distinguished. A corpus albicans from the previous menstrual cycle was also present in this ovary. No immunostaining was noted in the corpus albicans. No staining was observed in a third macaque ovary (no.78)(Plate 5.14) which contained many large follicles. This animal had been actively immunized against the first 1-23 amino acids of the N-terminus of the inhibin α-subunit. It is possible that the absence of staining could be explained by the immunization procedure, with the antigenic sites for inhibin being blocked by the anti-inhibin antibody.

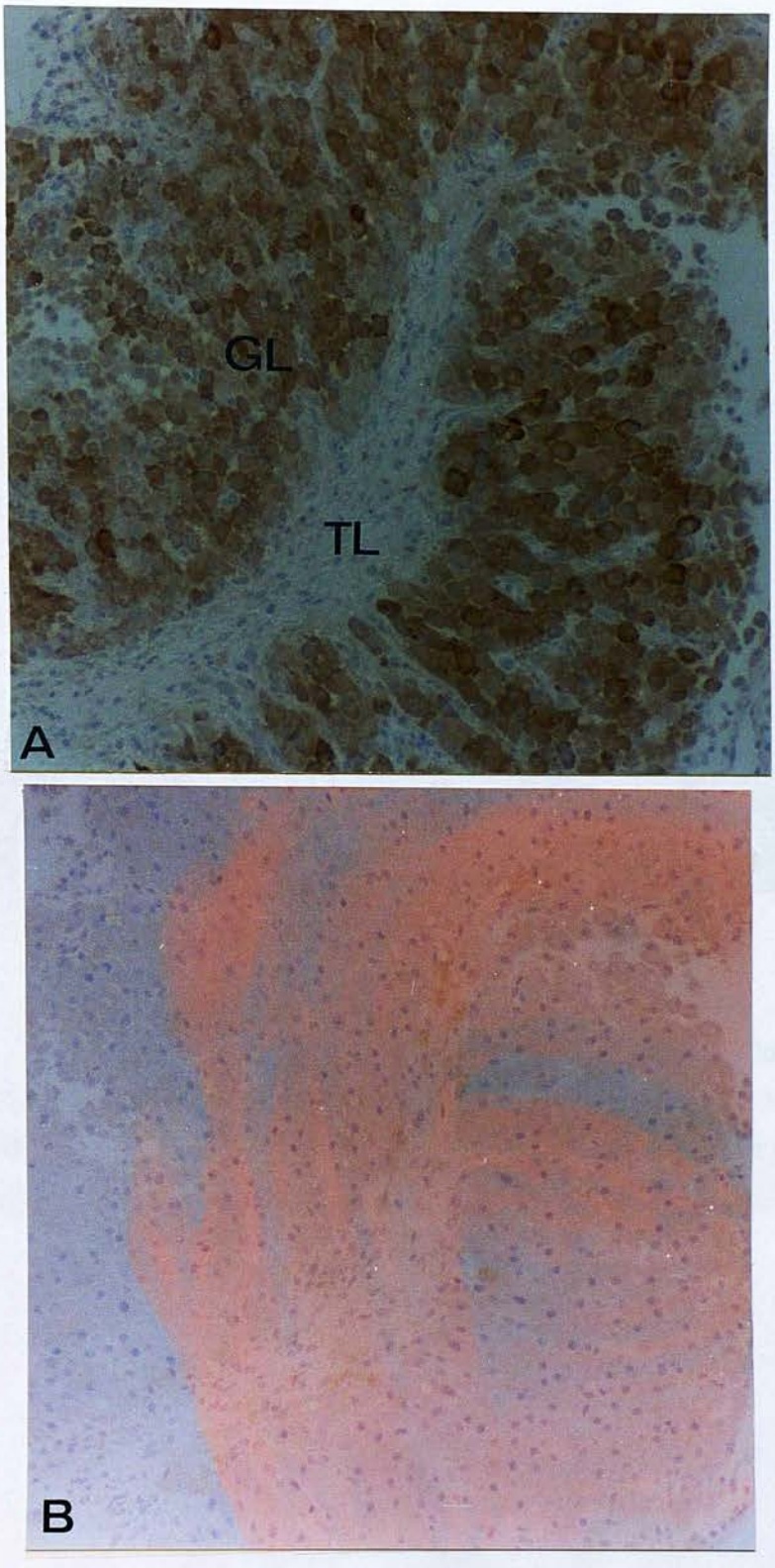


Plate 5.5. Immunocytochemical localization of immunoreactive inhibin in mid-luteal phase corpus luteum (A). Intense immunostaining is present within the granulosa-lutein cells (GL) with absence of staining in the intrusion of theca-lutein cells (TL). Note variation of intensity of staining within the GL cell population. In section B, the polyclonal antiserum was preabsorbed with excess inhibin peptide immunogen. No immunostaining is present. (x 33).

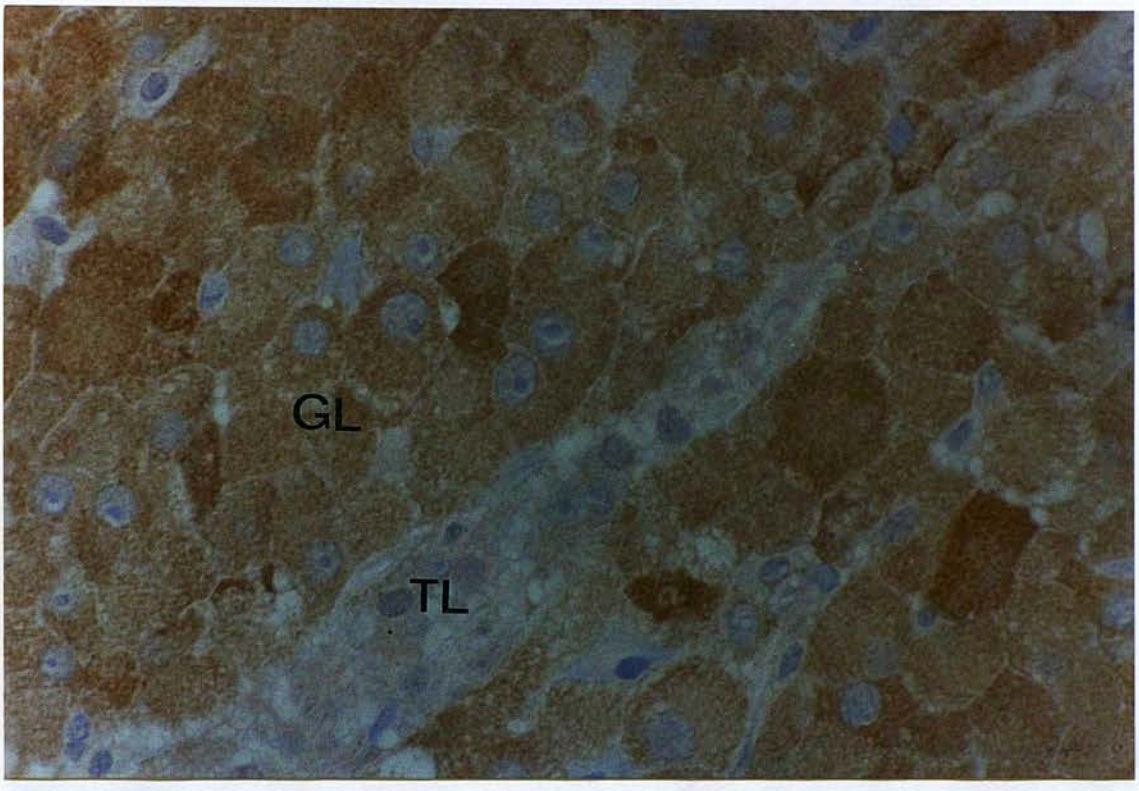
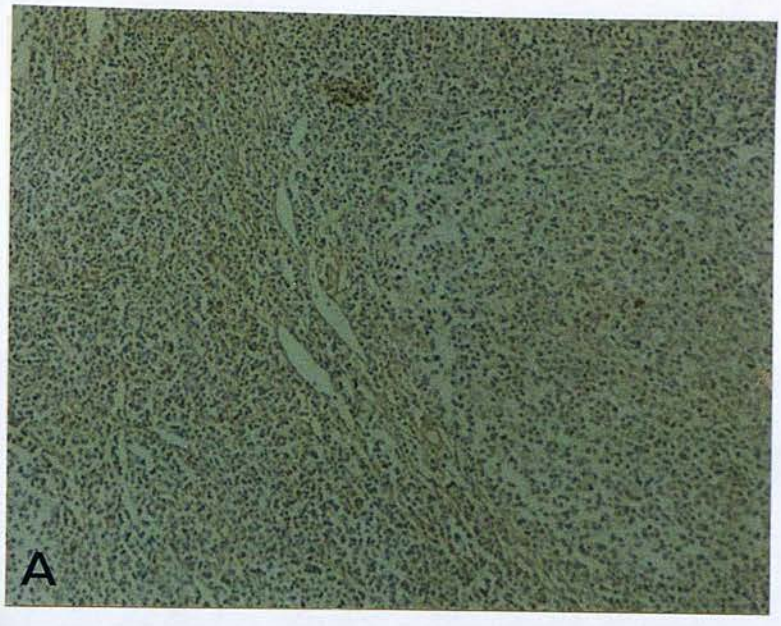


Plate 5.6. High magnification of mid-luteal phase human corpus luteum showing immunocytochemical localization of inhibin. Intense immunostaining is present in the granulosa-lutein cells (GL) with absence of staining in the intrusion of theca-lutein cells (TL) (x 132).



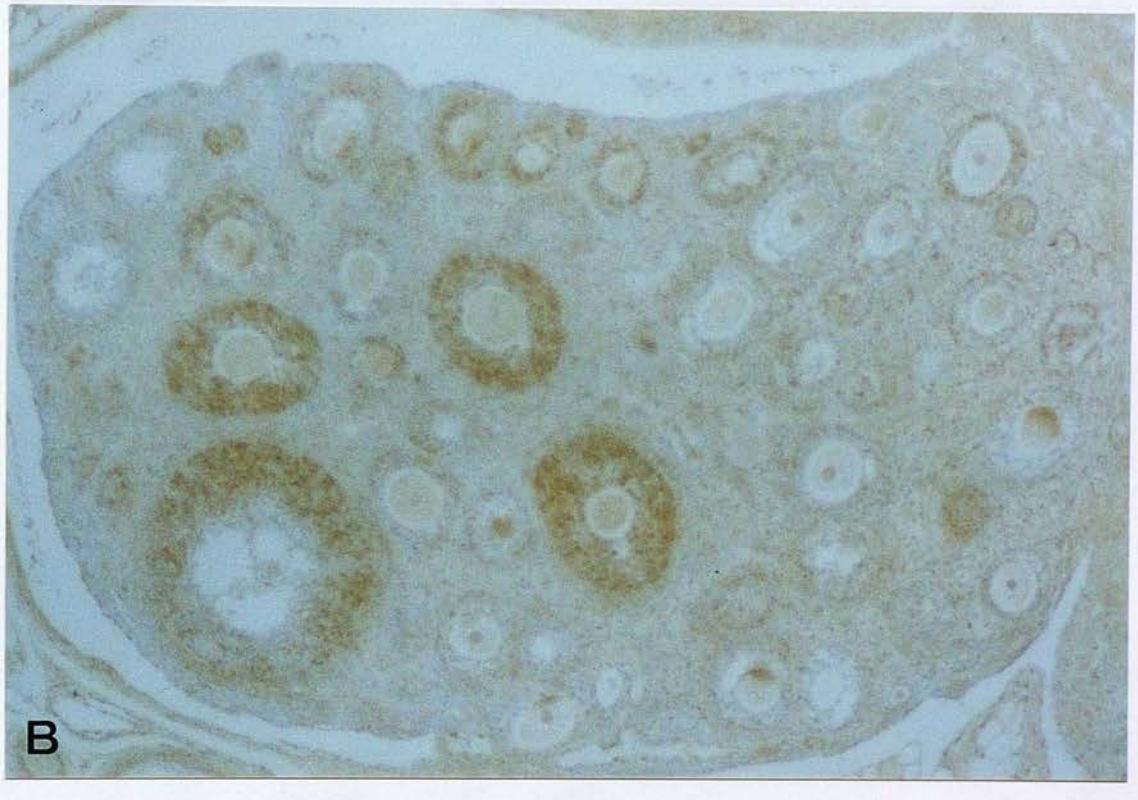


Plate 5.7. Human tonsil (A) was used as a negative control tissue and no immunostaining was obtained (x 25). Positive immunostaining for inhibin α-subunit was obtained within antral follicles from a immature rat ovary (B) (x 25).

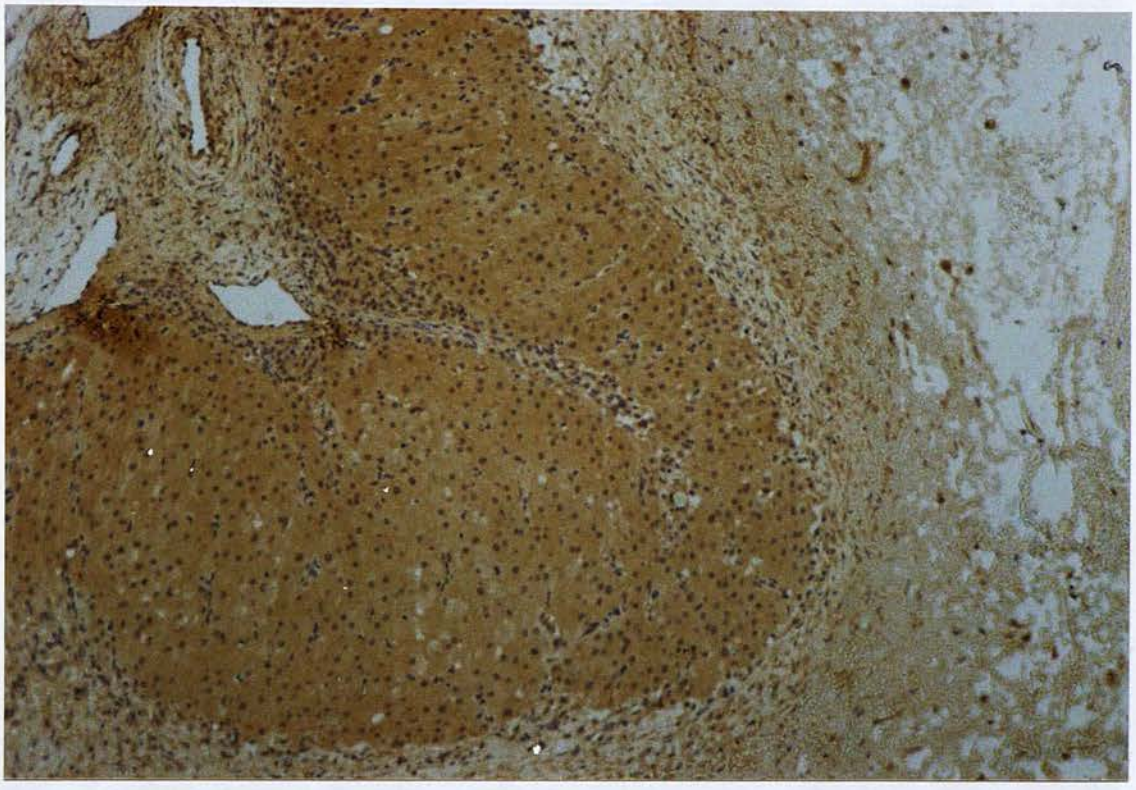
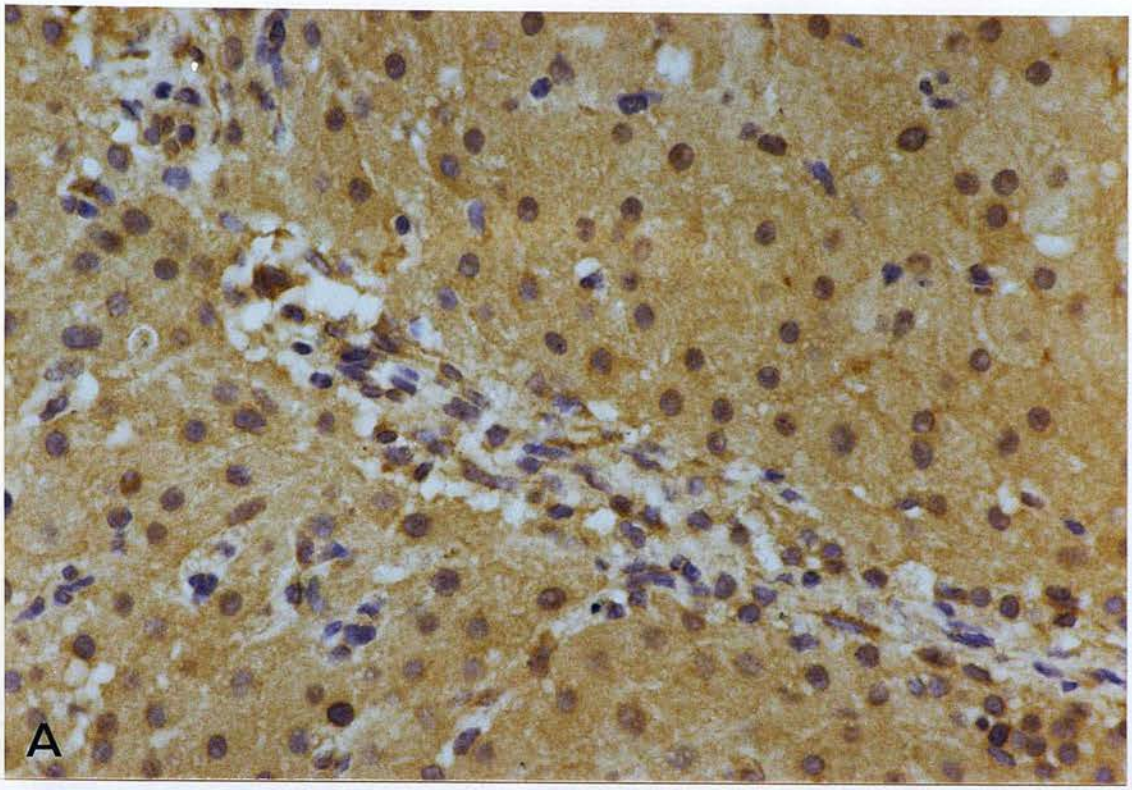


Plate 5.8. Immunocytochemical localization of inhibin β_A-subunit within the human corpus luteum (x 20).



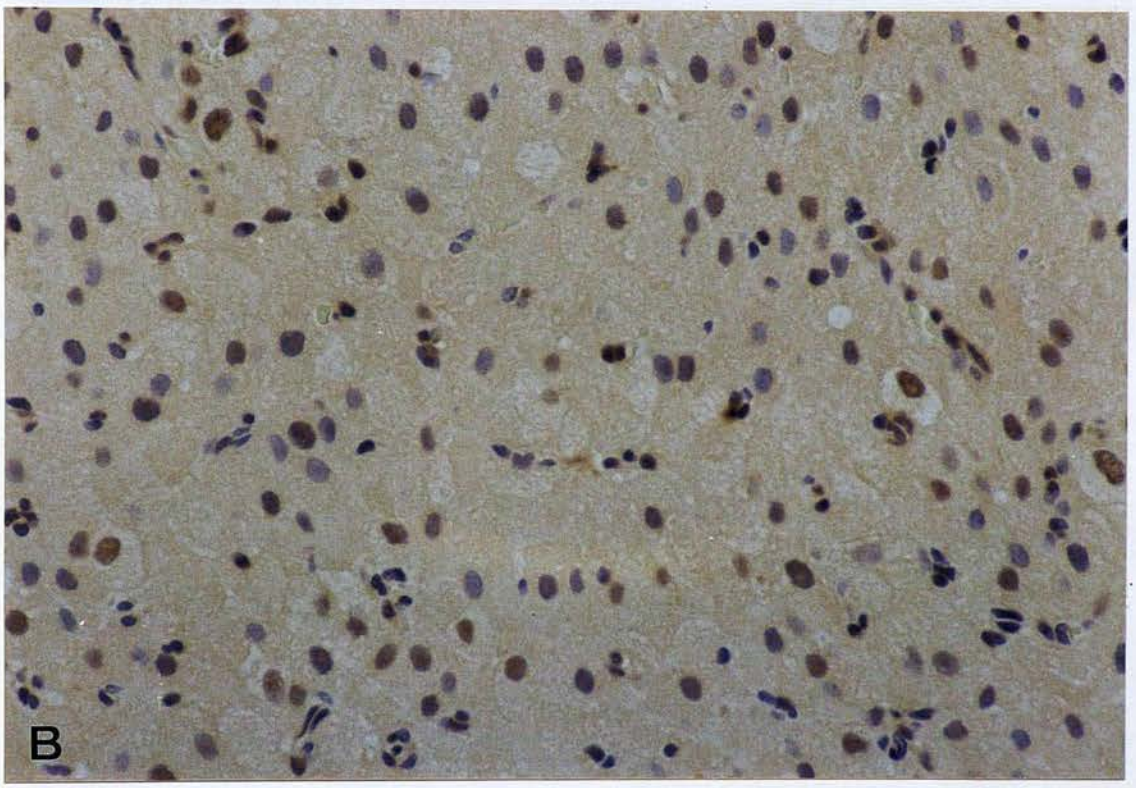


Plate 5.9. High magnification (x 40) of immunocytochemical localization of inhibin β_A-subunit in the human corpus luteum (A). Negative staining was obtained when primary antibody was substituted with normal mouse serum (B) (x 40).

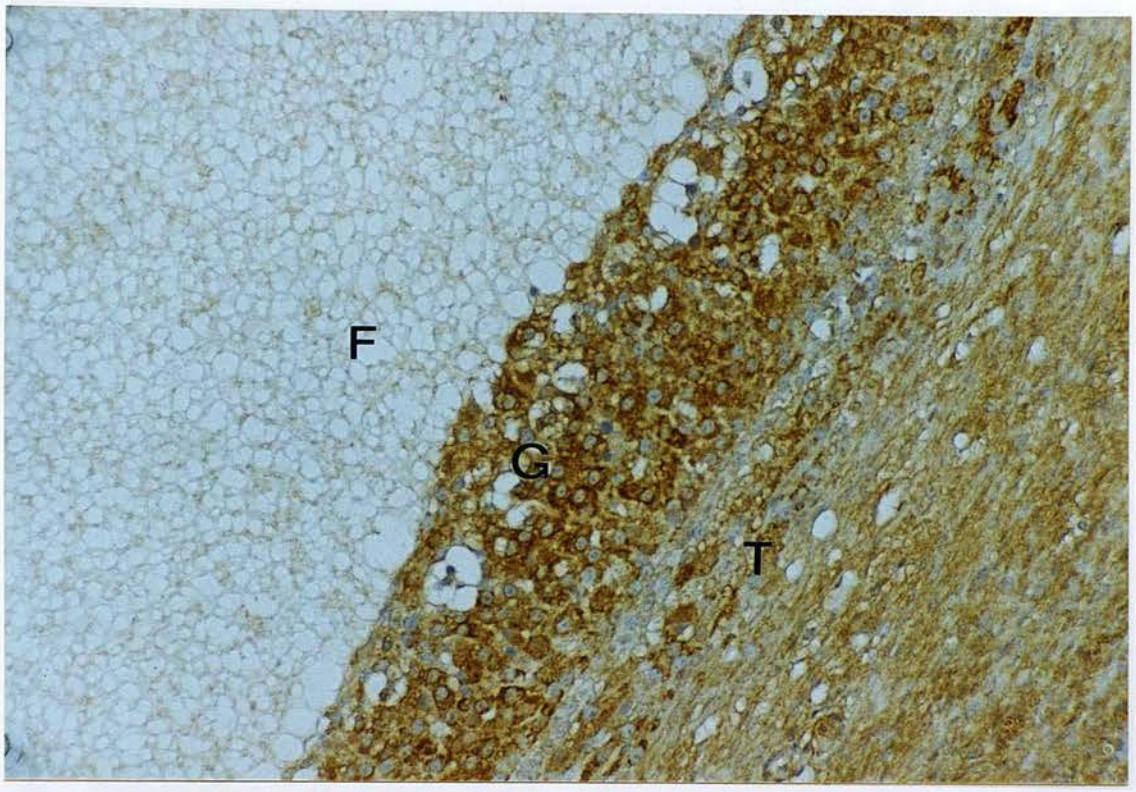


Plate 5.10. Immunocytochemical localization of inhibin α-subunit within the granulosa cell layers (G) of a human antral follicle with absence of staining in the theca cell layer (T). The follicular cavity is denoted by F. Positive staining was obtained in one out of five follicles studied (x 50).

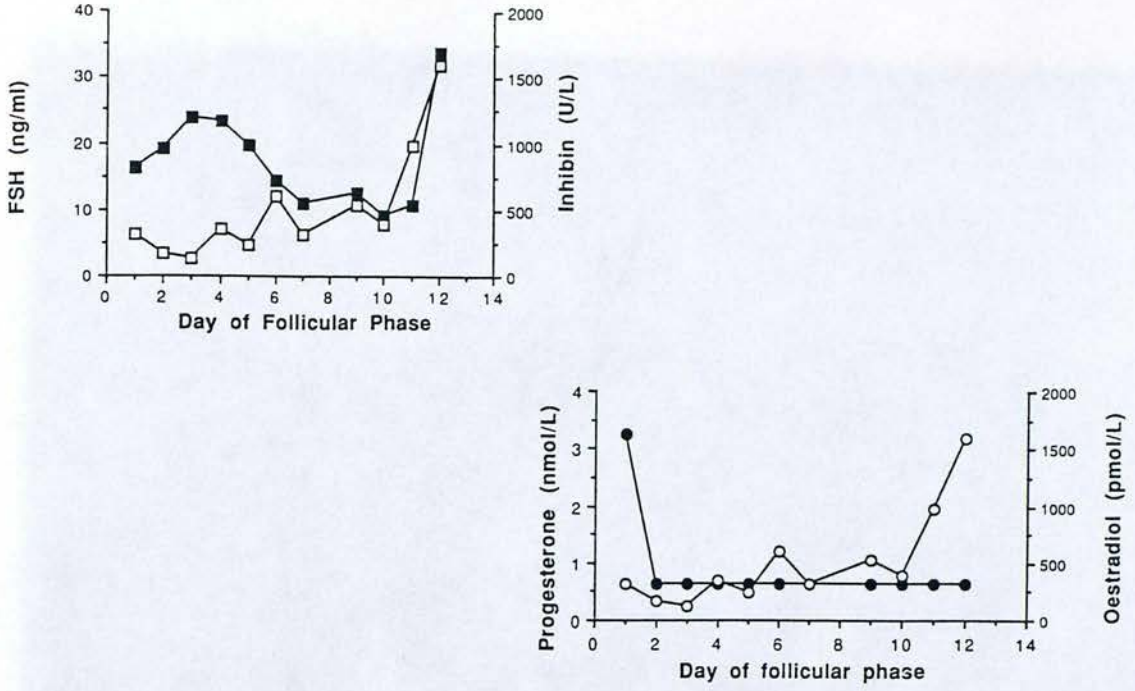


Fig. 5.5. Serum concentrations of progesterone (●), oestradiol (O) (A), FSH (■) and inhibin (□) (B) during the follicular phase prior to removal of the large macaque preovulatory follicle shown in plate 5.11.

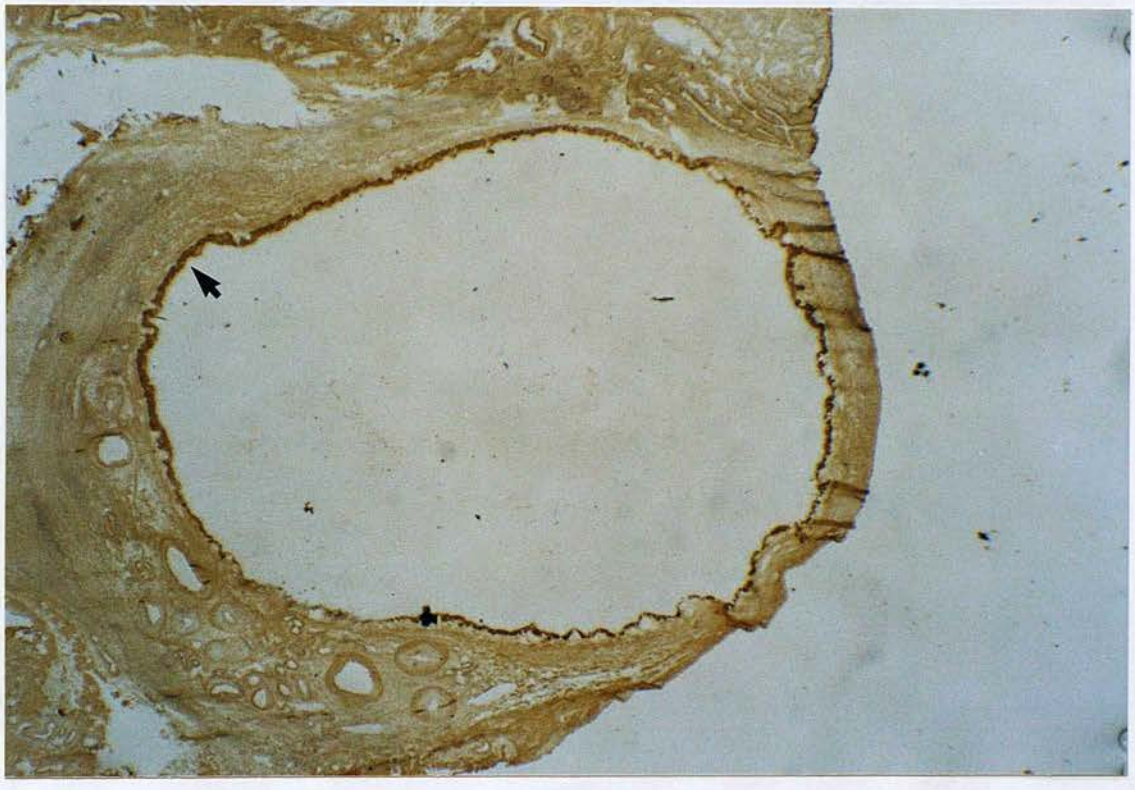


Plate 5.11. Immunocytochemical localization of inhibin α-subunit within the granulosa cell layer of a large preovulatory macaque follicle (arrow) (x 3.3).

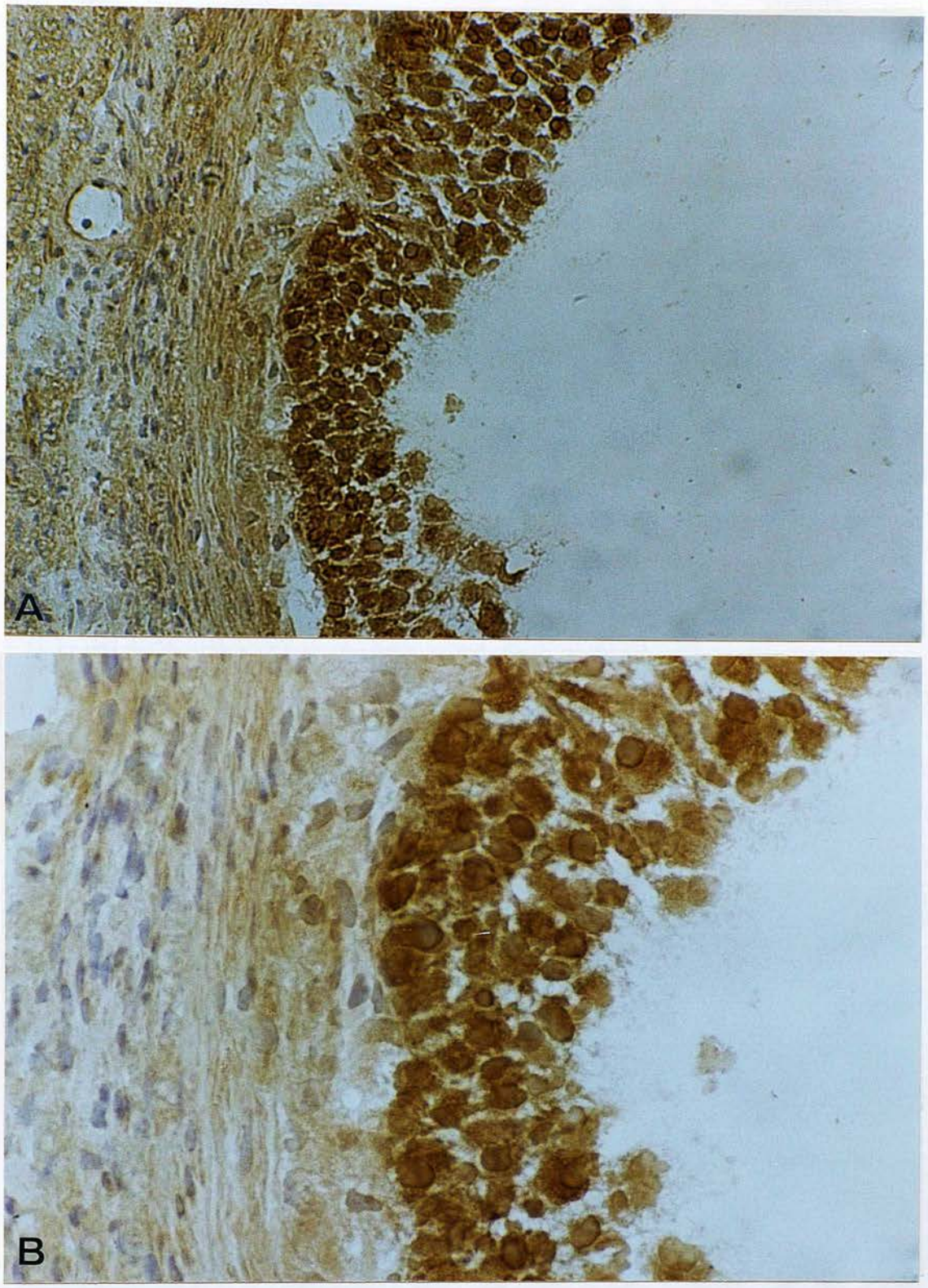
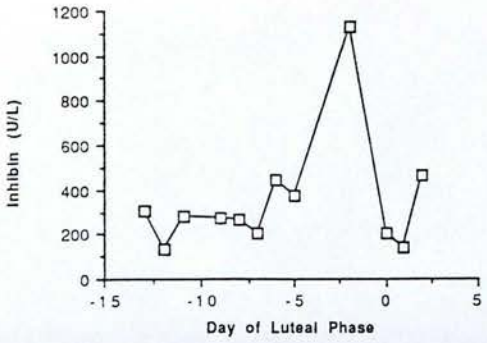


Plate 5.12. Higher magnification of immunocytochemical localization of inhibin α-subunit in macaque preovulatory follicle (A, x 67; B, x 132).



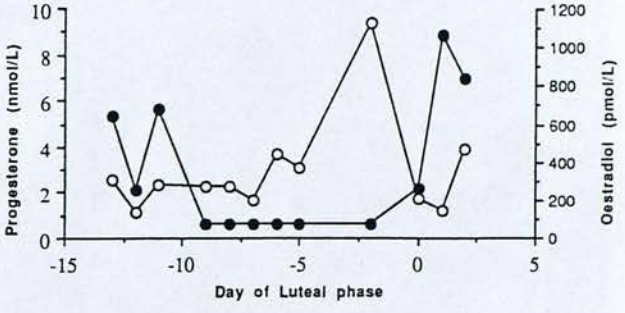


Fig. 5.6. Serum concentrations of progesterone (●), oestradiol (O) (A) and inhibin (□) (B) prior to removal of macaque ovary shown in plate 5.13.

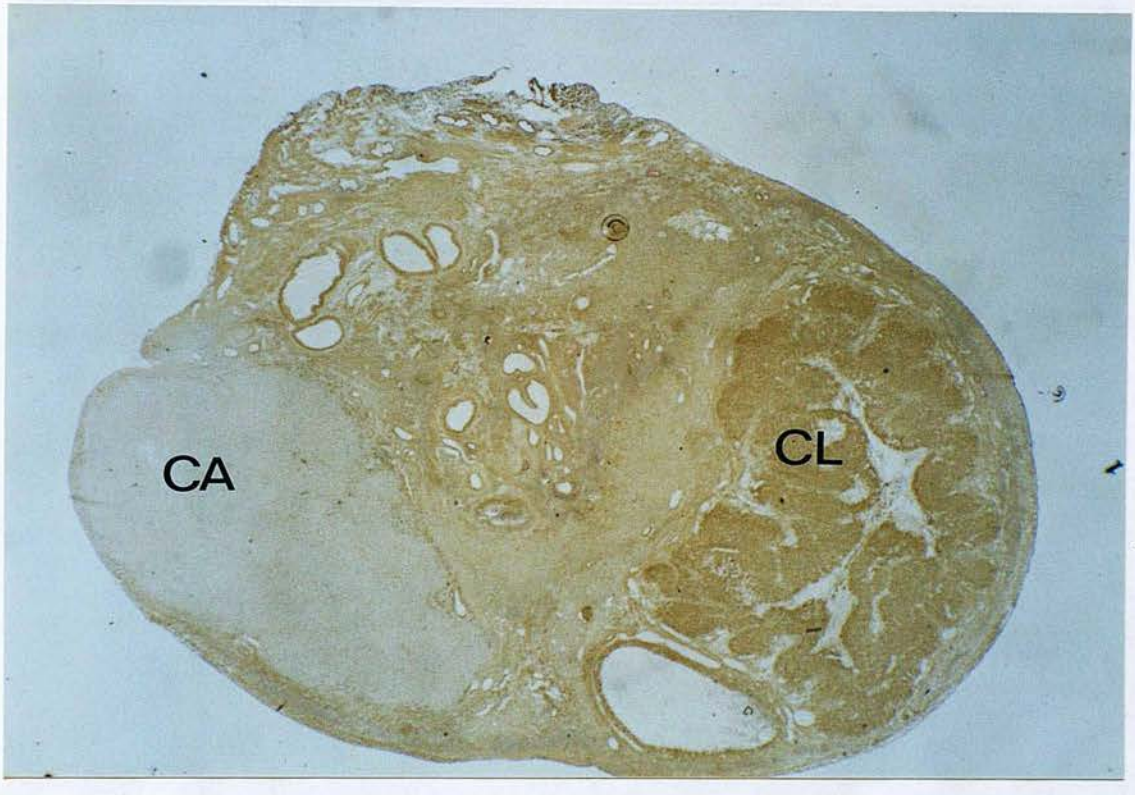


Plate 5.13. Immunocytochemical localization of inhibin α-subunit in macaque corpus luteum (CL). No immunostaining is present in the corpus albicans (CA) (x 2.5).

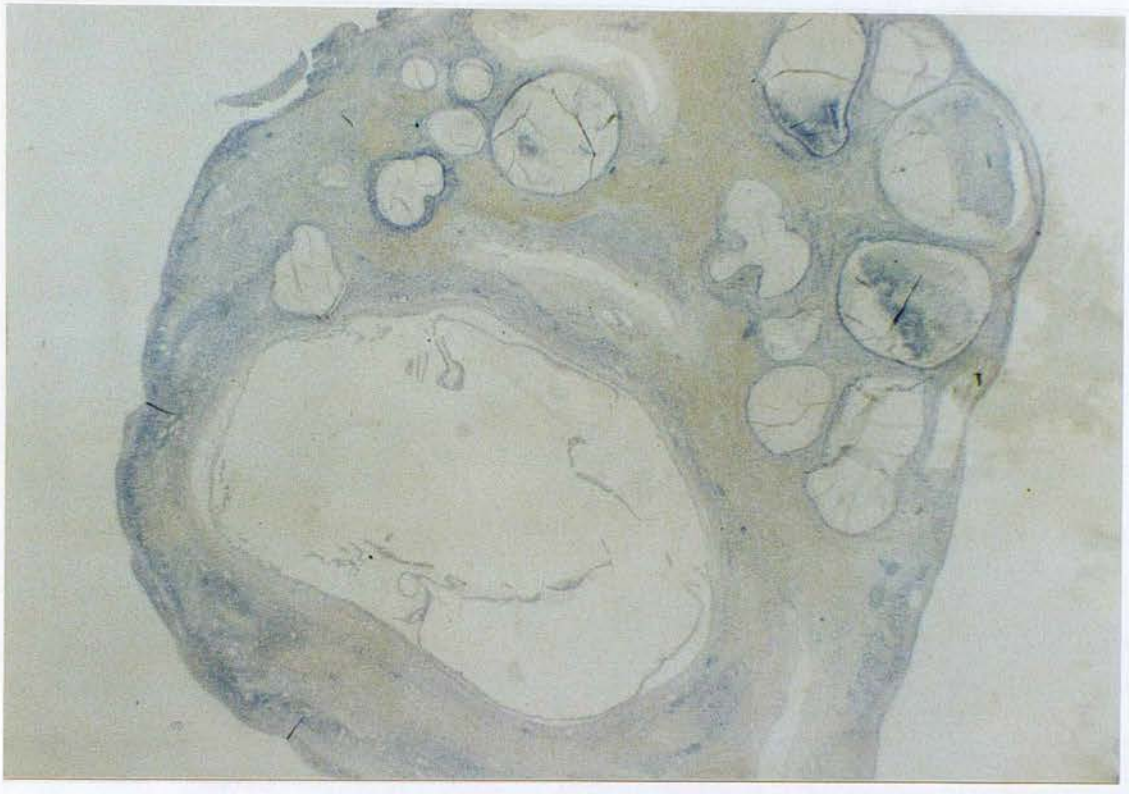


Plate 5.14. Low power section of macaque ovary (x 2.5) from an animal actively immunized against inhibin α-subunit. No immunostaining is present. Note large numbers of follicles in ovary.

5.2.4 Discussion

These results are consistent with the concept that the corpus luteum is a major source of immunoreactive inhibin during the primate menstrual cycle. Immunostaining for inhibin α-subunit was present at all stages of the luteal phase studied in the human and in the early corpus luteum of the macaque. There were no apparent changes in the intensity of staining. This is in agreement with the observation that there were no differences in immunoreactive inhibin concentrations in luteal tissue homogenates from various stages of the luteal phase in cynomolgous monkeys (Basseti et al. 1990) and with the findings of Reddi et al.(1990b) demonstrating that expression of inhibin α-subunit mRNA was present throughout the lifespan of the corpus luteum and that no correlation was found between the level of expression and age of the tissue. The changes in serum concentrations of immunoreactive inhibin during the luteal phase may therefore be due to factors other than alterations in the rate of transcription and translation of the α-subunit gene. The absence of staining in the corpus albicans in the macaque ovary was not surprising and suggests that it is unable to produce immunoreactive inhibin at this degenerative stage. Since biologically active inhibin consists of a β-subunit combined to the α-subunit, the findings of immunocytochemical localization of β_A-subunit within the granulosa-lutein cells of the mid-luteal phase human corpus luteum, although preliminary, suggests that the corpus luteum may produce biologically active inhibin. Further studies are required to investigate the localization of β_A-subunit throughout the different stages of the luteal phase.

Inhibin α-subunit was localized in the granulosa cell layer of one small human antral follicle and in one macaque preovulatory follicle. The reason for the failure of staining in four out of five human antral follicles is unknown. Immunoreactive inhibin is present in high concentrations in follicular fluid (deJong & Sharpe, 1976; Schwartz & Channing, 1977; Chari et al., 1979) and is produced in culture by the granulosa cells of many species including man (Tsonis et al., 1987b). In the rat, inhibin α-subunit immunostaining was demonstrated in ovarian follicular granulosa cells as a positive control tissue as immunostaining for inhibin α-subunit had been demonstrated previously at all stages of maturation (Cuevas et al., 1987; Meunier et al., 1988a) .

Little is known about the factors regulating the production of inhibin by different follicles. In the rhesus monkey not all follicles express both α- and β-subunits necessary for the synthesis of inhibin (Schwall et al., 1990). In women and non-human primates, the concentration of immunoreactive inhibin during the follicular

phase of the cycle is much lower than during the luteal phase (McLachlan et al., 1987a; Fraser et al., 1989). Thus in the human it may be that only a minority of follicles produce immunoreactive inhibin in amounts sufficient to be detected by immunocytochemistry.

Inhibin α- and β_A- subunits were localized specifically in the large granulosa-lutein cells of the corpus luteum with no staining in the smaller theca-lutein cells. Similarly, P450 aromatase has also been demonstrated specifically in the granulosa-lutein cells of the human corpus luteum whereas P450 17α-hydroxylase was localized in the theca-lutein cells (Sasano et al.,1989) suggesting that the secretion of oestradiol by the corpus luteum may be dependent on aromatization in the granulosa-lutein cells of androgen substrate produced by theca-lutein cells. The specific cellular localization of inhibin α-subunit may suggest that immunoreactive inhibin is also involved in a paracrine mechanism within the corpus luteum as well as its putative endocrine role in suppressing pituitary FSH secretion. Current evidence suggests that immunoreactive progesterone and inhibin secretion into the peripheral circulation during the luteal phase is under the control of LH. In chapter 3, treatment with LH- releasing hormone antagonist for 3 days starting during the mid-luteal phase in the stumptailed macaque caused a permanent suppression of serum immunoreactive progesterone and inhibin concentrations for the remainder of the cycle. Furthermore, in chapter 3 human chorionic gonadotrophin but not follicle-stimulating hormone could overcome the inhibitory effects of the gonadotrophin withdrawal by the antagonist in the stumptailed macaque. However, the significance of the gonadotrophic control of progesterone and inhibin and the paracrine role which immunoreactive inhibin may play within the corpus luteum remains to be investigated.

Chapter 6

General Discussion- The source of Inhibin secretion and its physiological role

6.1 Endocrine Role

Although the concept of 'inhibin' as a substance which selectively suppresses FSH secretion has been recognised for over fifty years, it is only within the past 5 years following the elucidation of the structure in 1985 and the development and expansion of techniques available for investigation, that significant advances have been made in determining the physiological properties of this glycoprotein. The studies described in this thesis have investigated the endocrine role of inhibin and the control of inhibin secretion using LHRH analogues and gonadotrophins *in vivo,* hCG on luteal cells in *in vitro* and finally immunocytochemistry, to determine the cellular localization of inhibin within the primate ovary.

Both the stumptailed macaque and the common marmoset monkey demonstrated elevations of inhibin levels during the luteal phase similar to the normal human menstrual cycle, suggesting that the corpus luteum is a major source of inhibin secretion (Chapter 2). The observations from the clinical studies of higher concentrations of inhibin in the vein draining the corpus luteum and of a fall in the peripheral concentration of inhibin after luteectomy, provide further evidence for the secretion of inhibin into the circulation by the corpus luteum (Chapter 2). It is tempting to speculate from these results that inhibin plays an endocrine role in the suppression of FSH during the luteal phase. It is already known that oestradiol contributes to the suppression of FSH at this time because administration of anti-oestrogens such as clomiphene or tamoxifen (Lumsden et al., 1989) or antiserum to oestradiol (Zeleznik et al., 1987) results in a rise in the circulating FSH concentrations. The suppression of FSH during the luteal phase may therefore be due to the synergistic effects of both inhibin and oestradiol. Studies involving passive immunization against inhibin during the luteal phase will provide further information on the role of inhibin at this time.

During the follicular phase, the concentration of inhibin in venous plasma draining the dominant follicle is no higher than that draining the contralateral side (Chapter 2). It therefore seems unlikely that the secretion of inhibin by the dominant follicle is responsible for the fall in FSH concentrations at this stage of the cycle. As inhibin suppresses the secretion of FSH selectively without affecting LH, it might be expected

that its concentration would rise during the follicular phase of the cycle as the secretion of FSH falls. However there is no change in the concentration of bioactive or immunoactive inhibin during the follicular phase of the cycle until within 2 days of ovulation, at the time of the midcycle surge of FSH and LH (Baird et al., 1990). The negative correlation between the concentration of oestradiol and the concentration of FSH suggests rather that oestradiol plays a dominant role at this stage of the cycle (Tsonis et al., 1987a). As demonstrated in chapter 4, it requires the growth of multiple antral follicles to induce marked rises in inhibin secretion in the blood during follicular development as in the nonphysiological situation of hyperstimulation regimens.

6.2 Paracrine Role

High concentrations of inhibin are present in human follicular fluid (Channing et al., 1984). In sheep, inhibin concentrations in follicular fluid vary from 2000 to 29000 U/L, being highest in oestrogenic follicles of greater than 3mm in diameter, whereas inhibin levels in human follicular fluid are lower by one to two orders of magnitude (Baird et al., 1988). Baird et al. (1990) have separated inhibin in human follicular fluid by gel filtration using a high-resolution Sephacryl gel matrix and have identified two peaks of immunoactive inhibin corresponding to approximate molecular weights of 32kD and 110kD, both of which are biologically active. The presence of high concentrations of inhibin in human follicular fluid suggests that inhibin may have a paracrine role during the follicular phase of the menstrual cycle.

In the rat, FSH was shown to be the predominant endocrine regulator of the production of inhibin by granulosa cells *in vitro*, while LH at low doses was able to cause a release of inhibin after exposure to FSH (Bicsak et al., 1986; Zhiwen et al., 1988a). Inhibin is produced by human granulosa cells in culture *in vitro* and its release is stimulated by FSH and LH (Tsonis et al., 1987b). The stimulation of inhibin production by granulosa cells from marmoset and women by FSH is further enhanced by both testosterone and oestradiol (Hillier et al., 1989). Insulin-like growth factor -1 (IGF-1), either alone or in synergism with FSH, stimulated inhibin production by rat granulosa cells in a time- and dose-dependent manner (Bicsak et al., 1986; Zhiwen et al., 1987a) whereas epidermal growth factor (EGF) inhibited FSH-stimulated production of inhibin by rat granulosa cells *in vitro* (Bicsak et al., 1986; Franchimont et al., 1986; Zhiwen et al., 1987b). Therefore, while FSH induces differentiation of the functions of the granulosa cell eg. aromatase and inhibin secretion, these actions are further regulated in the granulosa cell by paracrine factors.

The specific localization of inhibin within the granulosa-lutein cells of the corpus luteum (chapter 5) suggests that inhibin may also have a paracrine role during the luteal phase. P_{450} aromatase enzyme has also been localized immunocytochemically within the granulosa-lutein cells of the human corpus luteum. This suggests that the luteinized granulosa-lutein cells of the primate corpus luteum retain the specialized functions present before the transformation from follicle to corpus luteum following ovulation such as the 'two cell-two gonadotrophin theory' of oestradiol synthesis and the presence of inhibin within the granulosa cells. The significant homology of inhibin with substances such as TGF-β may suggest that the role of inhibin extends far beyond the original proposed role of selective suppression of FSH. Both inhibin and activin have actions on erythropoiesis (Eto et al., 1987; Yu et al., 1987) and thymocyte cell division (Hedger et al., 1989). The follicle and corpus luteum are dynamic tissues and their structures change rapidly over the course of the menstrual cycle. It could be speculated that inhibin and activin could have a role to play in cellular growth and differentiation in the ovary.

6.3 Control of Inhibin secretion

6.3.1 Follicular Phase

It has recently been demonstrated that the capacity of human granulosa cells to produce immunoreactive inhibin *in vitro* is developmentally regulated (Hillier et al., 1991a). Initially FSH acts directly to induce inhibin production and steroidogenesis in nondifferentiated granulosa cells and this response is modulated by androgen. However, as these cells undergo preovulatory development, the production of inhibin and progesterone becomes increasingly responsive to direct stimulation by LH. These findings are consistent with the idea that postovulation, granulosa-lutein cells are sites of LH-responsive inhibin as well as steroid synthesis in the corpus luteum.

6.3.2 Luteal Phase

The findings in chapter 3 that hCG, but not FSH, was able to overcome LHRH antagonist-induced suppression of luteal function in the stumptailed macaque has recently been demonstrated in women (McLachlan et al., 1989). Furthermore, the finding that inhibin production by human luteal cells *in vitro* could be stimulated by hCG was demonstrated in luteinized granulosa cells in culture by Tsonis et al. (1987b). These findings taken together with the results in chapter 2, where the human corpus luteum responded to stimulation with hCG by producing significantly increased amounts of inhibin, suggest that the secretion of inhibin by the corpus luteum is LH-dependent. In a recent study, Nakajima et al.(1990) demonstrated that mid-luteal

immunoreactive inhibin levels, similar to LH and progesterone, exhibited a pulsatile pattern. However, the inhibin secretory pattern was more rapid and of lower amplitude in comparison with progesterone. Although luteal inhibin concentrations parallel progesterone levels (McLachlan et al., 1987a), the results indicated only a loose association between inhibin and progesterone secretory patterns and analysis of intermittent hormone pulses did not suggest a coupled secretion pattern. These findings are in agreement with a study in the ewe (McNeilly & Baird, 1989) which reported pulsatile inhibin release from preovulatory follicles was unrelated to pulses of LH or oestradiol. Thus, it is possible that a different mechanism exists for LH-dependent luteal inhibin production in comparison with progesterone.

6.3.3 Periovulatory Phase

McLachlan et al. (1990) examined the secretion of inhibin during the periovulatory phase in detail. They concluded that the maturing follicle secretes both oestradiol and inhibin in parallel until 18h before ovulation, at which time the process of luteinization is initiated by the onset of the LH surge. Oestradiol secretion then falls while inhibin secretion rises indicating different regulation of secretion of these hormones by the maturing follicle. Furthermore, the close positive correlation between inhibin and gonadotrophin levels around midcycle suggests that FSH and/or LH stimulates inhibin secretion and that the presumed negative feedback effect of inhibin on FSH secretion is overcome at this time. After midcycle, inhibin secretion initially falls then rises while progesterone rises progressively. This transient divergence of progesterone and inhibin secretion may occur during the transformation of the preovulatory follicle into the corpus luteum. The rise in inhibin concentrations in serum at mid-cycle may also be related to the breakdown of the basement membrane between the granulosa and theca layers under the action of the LH surge. Inhibin is present in concentrations 50-100-fold higher in follicular fluid than in ovarian venous blood and hence there must be some barrier present to prevent the free passage of inhibin out of the follicle.

It has been demonstrated that androgens stimulate inhibin secretion by granulosa cells *in vitro* (Henderson & Franchimont, 1981; Tsonis et al., 1987b) and also that androgen levels decline in human follicular fluid in the days leading up to ovulation (McNatty et al., 1976). Furthermore, in primate ovaries, mRNA for inhibin/activin β_B subunit is expressed in greatest amounts by granulosa cells in small antral follicles decreasing to undetectable levels in preovulatory follicles (Schwall et al., 1990). A decline in androgen stimulation of granulosa cell inhibin secretion may therefore be a component in the transient decline of inhibin levels immediately after the LH surge. In

contrast, recombinant human activin A causes a dose-dependent inhibition of androgen production by human thecal cells *in vitro* (Hillier et al. 1991b). Thus it is possible that a loop of paracrine interactions controls the production of inhibin during follicular development i.e. androgens stimulate inhibin production while the formation of activin inhibits androgen production.

6.4 Clinical Applications in Women

Inhibin measurements have been demonstrated to be useful in evaluating a variety of pathophysiological conditions. Serum inhibin concentrations have been shown to be a useful marker of the trophoblastic disease, hydatidiform mole (Yohkaichiya et al., 1989). Inhibin levels were much higher in patients with this disease than in normal pregnant women and inhibin was found to be a more specific marker for such tumours in comparison with hCG, with inhibin concentrations declining to follicular phase values in less than 10 days if removal of molar tissue was complete. Inhibin has also been shown to be secreted in abnormally high concentrations in women with granulosa cell tumours (Lappohn et al., 1989). Since serum inhibin levels correlated with the size of the tumour, measurements of inhibin may be used as a marker for primary as well as recurrent disease. Soules et al. (1989) have shown that inhibin secretion during the follicular phase of cycles in women with luteal phase deficiency is low which suggests that the origin of luteal phase deficiency may lie in defective folliculogenesis. Several investigators have proposed that inhibin may play a role in producing the high LH:FSH ratio found in women with polycystic ovarian disease which was supported by the finding of high bioactive inhibin levels in the follicles of women with this disease (Tanabe et al., 1983). However, in an extensive study by Buckler et al. (1988), they could find no difference in serum inhibin concentrations in women with polycystic ovarian disease, regardless of the stage of their cycle. Furthermore, the substance which was found to cross-react in the inhibin assay from subject X in the endometriosis study (chapter 4) may prove to be an inhibin-like peptide and may be related in some way to the pathology of the disease or infertility.

6.5 The Measurement of Inhibin and Future Prospects

The existence of non-bioactive inhibin-like forms such as pro-αC and free α-subunit, which have been identified in bovine follicular fluid, have complicated the measurement of biologically active inhibin due to cross reaction of these substances in the available radioimmunoassays. However, these forms of inhibin have not been detected in biological fluids in primates. It is now necessary to develop specific assays with antisera directed to epitopes on both α-and β-subunits (two-site assays) to ensure

that only whole bioactive inhibin is being detected. A universal standard preparation is not available at present and this is essential to enable comparison of immunoreactive inhibin levels between studies performed by different research groups throughout the world. Research into the physiology of inhibin has been hampered further by the difficulty in obtaining sufficient quantities of purified inhibin for experimental purposes. The molecular species of inhibin in human peripheral blood and ovarian vein blood is unknown, as is the half-life and metabolism of inhibin. The inhibin receptor, most likely to be present in pituitary and ovary, has yet to be characterized.

Although the measurement of inhibin immunoreactivity may be used as a reliable marker for detecting pathophysiological conditions, the administration of inhibin as a contraceptive agent by suppressing FSH is unlikely at present, from both a chemical and physiological point of view, due to the size of the molecule and the wide range of other actions this peptide may have which may lead to unwanted side-effects. It is possible that inhibin or inhibin-like forms might be used for veterinary purposes as active immunization of sheep against the α_c subunit, which produced antibodies recognizing bioactive inhibin, increased FSH production (Findlay et al., 1989a), ovulation rate (Forage et al., 1987) and lambing rate (Tsonis et al., 1989) whereas immunization against the α_n-subunit in the sheep has been shown to result in reduced fertility (Findlay et al., 1989b). The recent isolation of recombinant human inhibin A from mammalian cell culture media (Tierney et al., 1990) and its application in both *in vivo* and *in vitro* experiments, will help to increase our knowledge of the endocrine and paracrine actions of inhibin and its related peptides.

Bibliography

Abraham, GE, Odell, W.D. Swerdloff, R.S. & Hopper, K. (1972) Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17α-hydroxyprogesterone and estradiol-17β during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **34,** 312-318

Adams, E.C. & Hertig, A.T. (1969). Studies of the human corpus luteum. I. Observations of the ultrastructure of development and regression of the luteal cells during the menstrual cycle. *Journal of Cell Biology* **41**, 696-715.

Adams, L.A., Bremner, W.J., Nestor, J.J., Vickery, B.H. & Steiner, R.A. (1986). Suppression of plasma gonadotrophins and testosterone in adult male monkeys (*Macaca fascicularis*) by a potent inhibitory analog of gonadotrophin-releasing hormone. *Journal of Clinical Endocrinology and Metabolism* **62**, 58-63.

Adams, T.E., Norman, R.L. & Spies, H.G. (1981). Gonadotrophin-releasing hormone receptor binding and pituitary responsiveness in estradiol-treated monkeys. *Science* **213**, 1388-1390

Akhtar, B.F, Marshall, G.R., Wickings, E.J. & Nieschlag, E. (1983). Reversible induction of azoospermia in rhesus monkeys by constant infusion of a gonadotrophin-releasing hormone agonist using osmotic minipumps. *Journal of Clinical Endocrinology and Metabolism* **56**, 534-540.

Alila, H.W., Corradino, R.A. & Hansel, W. (1988a). A comparison of the effects of cycloxygenase prostanoids on progesterone production by small and large bovine luteal cells. *Prostaglandins* **36**(2), 259-267.

Alila, H.W., Dowd, J.P., Corradino, R.A., Harris, W.V. & Hansel, W. (1988b). Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. *Journal of Reproduction and Fertility* **82** (2), 645-655.

Amico, J.A., Ervin, M.G., Leake, R.D., Fisher, D.A., Finn, F.M. & Robinson, A.G. (1985). A novel oxytocin-like and vasotocin-like peptide in human plasma after administration of oestrogen. *Journal of Clinical Endocrinology and Metabolism* **60**(1), 5-12.

Amico, J.A., Seif, S.M. & Robinson, A.G. (1981). Elevation of oxytocin and the oxytocin associated neurophysin in the plasma of normal women during midcycle. *Journal of Clinical Endocrinology and Metabolism* **53**, 1229-1232.

Arimura, A., Kastin, A.J., Gonzalez-Barcena, D., Siller, J., Weaver, R.E. & Schally, A.V. (1974). Disappearance of LH-releasing hormone in man as determined by radioimmunoassay. *Clinical Endocrinology* **3**, 421-425.

Auletta, F.J., Caldwell, B.V., van Wagenen, G. & Morris, J.M. (1972). The effect of postovulatory oestrogen on progesterone and prostaglandin F levels in the monkey. *Contraception* **6**, 411-421.

Auletta, F.J., Agins, H. & Scommegna, A. (1978). Prostaglandin F mediation of the inhibitory effect of oestrogen on the corpus luteum of the rhesus monkey. *Endocrinology* **103**, 1183-1189.

Auletta, F.J. & Flint, A.P.F. (1988). Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates and women especially in relation to the time of luteolysis. *Endocrine Reviews* **9** (1), 88-105.

Auletta, F.J., Jones, D.S.C. & Flint, A.P.F. (1988b). Does the human corpus luteum synthesize neurohypophysial hormones? *Journal of Endocrinology* **116**, 163-165.

Auletta, F.J., Kamps, D.L., Wesley, M. & Gibson, M. (1984a). Luteolysis in the rhesus monkey: Ovarian venous oestrogen, progesterone and prostaglandin $F_{2\alpha}$ metabolite. *Prostaglandins* **27**, 299-310.

Auletta, F.J., Paradis, D.K. & Gibson, M. (1985). Luteolysis in the rhesus monkey: effect of chronic intra-corpus luteum infusion of oestrogens on luteal function. *Fertility and Sterility* Abstract P-137, p127.

Auletta, F.J., Paradis, D.K., Wesley, M. & Duby, R.T. (1984b). Oxytocin is luteolytic in the rhesus monkey (*Macaca mulatta*). *Journal of Reproduction and Fertility* **72**, 401-406.

Auletta, F.J., Robertson, L.M. & Schofield, M.J. (1988a). Luteal maintenance in the rhesus monkey: effect of ibuprofen and human chorionic gonadotrophin. 8th International Congress of Endocrinology, Kyoto, Japan. Abstract 454.

Auletta, F.J., Schofield, M.J. & Abae, M. (1990). The mechanisms controlling luteolysis in non-human primates and women. *Seminars in Reproductive Endocrinology* **8** (2), 122-129.

Auletta, F.J., Speroff, L. & Caldwell, B.V. (1973). Prostaglandin $F_{2\alpha}$-induced steroidogenesis and luteolysis in the primate corpus luteum. *Journal of Clinical Endocrinology and Metabolism* **36**, 405-407.

Backstrom, C.T., McNeilly, A.S., Leask, R.M. & Baird, D.T. (1982). Pulsatile secretion of LH, FSH, prolactin, oestradiol and progesterone during the human menstrual cycle. *Clinical Endocrinology* **17**, 29-42.

Baird, D.T., Fraser, H.M., Hillier, S.G., Illingworth, P.J., McNeilly, A.S., Reddi, K. & Smith, K. (1990). Production and secretion of ovarian inhibin in women. In '*Neuroendocrine Regulation of Reproduction in Women.*' pp 195-205. Eds. S.S.C.Yen & W.W.Vale. Serono Symposia U.S.A.

Baird, D.T. & Fraser, I.S. (1975). Concentration of oestrone and oestradiol in follicular fluid and ovarian venous blood of women. *Clinical Endocrinology* **4**, 259-266.

Baird, D.T., Tsonis, C.G., Messinis, I.E., Templeton, A.A., McNeilly, A.S. (1988). Inhibin levels in gonadotropin-treated cycles. *Annals of New York Academy of Sciences* **541**, 153-161.

Bardin, C.W., Morris, P.L., Chen, C.L., Shaha, C., Voglmayr, J., Rivier, J., Spiess, J. & Vale, W.W. (1987). Testicular inhibin: structure and regulation by FSH, androgens and EGF. In: *Inhibin Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion.* **42**: 179-190. Eds. H.G. Burger, D.M. de Kretser, J.K. Findlay & M. Igarashi. New York: Serono Symposia, Raven Press.

Basseti, S.G., Winters, S.J., Keeping, H.S. & Zeleznik, A.J. (1990). Serum immunoreactive inhibin levels before and after luteectomy in the cynomolgus monkey (*Macaca fascicularis*). *Journal of Clinical Endocrinology and Metabolism* **70**, 590-594.

Beard, A.J., Castillo, R.J., McLeod, B.J., Glencross, R.G. & Knight, P.G. (1990). Comparison of the effects of crude and highly purified bovine inhibin (Mr 32000) on plasma concentrations of FSH and LH in chronically ovariectomized prepubertal heifers. *Journal of Endocrinology* **125**, 21-30.

Beitins, I.Z. & Dufau, M.L. (1986). Pulsatile secretion of progesterone from the human corpus luteum: Poor correlation with bioactive LH pulses. *Acta Endocrinologica* **111**, 553-557.

Belchetz, P., Plant, T.M., Nakai, Y., Keogh, E.J. & Knobil, E. (1978). Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotrophin releasing hormone. *Science* **202**, 631-633.

Beling, C.G., Marius, S.L.& Markham, S.M. (1970). Functional activity of the corpus luteum following hysterectomy. *Journal of Clinical Endocrinology and Metabolism* **30**, 30-39.

Bennegard, B., Hahlin, M. & Dennefors, B. (1987). Antigonadotrophic effect of oxytocin on the isolated corpus luteum. *Fertility and Sterility* **47**, 431-435.

Bhattacharya, A.N., Dierschke, D.J., Yamaji, T. & Knobil, E. (1972). The pharmacologic blockade of the circhoral mode of LH secretion in the ovariectomized rhesus monkey. *Endocrinology* **90**, 778-786.

Bicsak, T.A., Cajander, S.B., Vale, W. & Hseuh, A.J.W. (1988). Inhibin: studies of stored and secreted forms by biosynthetic labelling and immunodetection in cultured rat granulosa cells. *Endocrinology* **122**, 741-748.

Bicsak, T.A., Tucker, E.N., Cappel, S., Vaughan, J., Rivier, J., Vale, W. & Hsueh, A.W. (1986). Hormonal regulation of granulosa cell inhibin biosynthesis. *Endocrinology* **119**, 2711-2719.

Boatman, D.E., Morgan, P.M. & Bavister, B.D. (1986) Variables affecting the yield and developmental potential of embryos following superstimulation and *in vitro* fertilization in rhesus monkeys. *Gamete Research* **13**, 327-338.

Bosu, W.T.K., Holmdahl, T.H., Johansson, E.D.B. & Gemzell, C.A. (1972) Peripheral plasma levels of oestrogens, progesterone and 17α-hydroxyprogesterone during the menstrual cycle of the rhesus monkey. *Acta Endocrinologica* **71**, 755-764

Bramley, T.A., Stirling, D., Swanston, I.A., Menzies, G.S., McNeilly, A.S. & Baird, D.T. (1987). Specific binding sites for gonadotrophin-releasing hormone, luteinizing hormone/chorionic gonadotrophin, low density lipoprotein, prolactin and follicle-stimulating hormone in homogenates of human corpus luteum. II. Concentrations throughout the luteal phase of the menstrual cycle and early pregnancy. *Journal of Endocrinology* **113**, 317-327.

Broxmeyer, H.E., Lu, L., Cooper, S., Schwall, R.H., Mason, A.J. & Nikolics, K. (1988). Selective and indirect modulation of human multipotential and erythroid hematopoietic progenitor cell proliferation by recombinant human activin and inhibin. *Proceedings of the National Academy of Sciences* **85**, 9052-9056.

Brunswig, B., Mukhopadhyay, A.K., Budnik, L.T., Bohnet, H.G. & Leidenberger, F.A. (1986). Phorbol ester stimulated progesterone production by isolated bovine luteal cells. *Endocrinology* **118**, 743-749.

Buckler, H.M., McLachlan, R.I., McLachlan, V.B., Healy, D.L. & Burger, H.G. (1988). Serum inhibin levels in polycystic ovary syndrome: basal levels in response to luteinizing hormone-releasing hormone agonist and exogenous gonadotrophin administration. *Journal of Clinical Endocrinology and Metabolism* **66**, 798-803.

Butler, W.R., Hotchkiss, J. & Knobil, E. (1975). Functional luteolysis in the rhesus monkey: ovarian oestrogen and progesterone during the luteal phase of the menstrual cycle. *Endocrinology* **96**, 1509-1512.

Caffrey, J.L., Nett, T.M., Abel, Jr.J.H. & Niswender, G.D. (1979). Activity of 3β-hydroxy-Δ^5 steroid dehydrogenase-Δ^{5-4} isomerase in the ovine corpus luteum. *Biology of Reproduction* **20**, 279-287.

Carmel, P.W., Araki, S. & Ferin, M. (1976). Pituitary stalk portal blood collection in rhesus monkeys: evidence for pulsatile release of gonadotrophin-releasing hormone (GnRH). *Endocrinology* **99**, 243-248.

Casper, R.F., & Cotterell, M.A. (1984). The effect of adrenergic and cholinergic agents on progesterone production by human corpus luteum tissue in organ culture. *American Journal of Obstetrics and Gynecology* **148**, 663-669.

Challis, J.R.G., Calder, A.A., Dilley, S., Forster, C.S., Hillier, K., Hunter, D.J.S., Mackenzie, I.Z. & Thorburn, G.D. (1976). Production of prostaglandins E and F by corpora lutea, corpora albicantes and stroma from the human ovary. *Journal of Endocrinology* **68**, 401-408.

Chambers, P.L. & Hearn, J.P. (1979) Peripheral plasma levels of progesterone, oestradiol-17β, oestrone, testosterone, androstenedione and chorionic gonadotrophin during pregnancy in the marmoset monkey, *Callithrix jacchus*. *Journal of Reproduction and Fertility* **56**, 23-32

Channing, C.P., Anderson, L.D., Hoover, D.G., Kolena, J., Osteen, K.G., Pomerantz, S.H. & Tanabe, K. (1982). The role of nonsteroidal regulators in control of oocyte and follicular maturation. *Recent Progress in Hormone Research* **38**, 331-340.

Channing, C.P., Chacom, M., Tanabe, K., Gagliano, P. & Tildon, T. (1984). Follicular fluid inhibin activity and steroid levels in ovarian tissue obtained at autopsy from human infants from 18 to 200 days of age. *Fertility and Sterility* **42**, 861-869.

Chari, S., Hopkinson, C.R.N., Darene, E. & Sturm, G. (1979). Purification of "inhibin" from ovarian follicular fluid. *Acta Endocrinologica* **90**,157-166.

Cheifetz, S., Weatherbee, J.A., Tsang, M.L.S., Anderson, J.K., Mole, J.E., Lucas, R. & Massague, J. (1987). The transforming growth factor-β-system, a complex pattern of cross-reactive ligands and receptors. *Cell* **48**, 409-415.

Chillik, C.F., Itskovitz, J., Hanh, D.W., McGuire, J.L., Danforth, D.R. & Hodgen, G.D. (1987). Characterizing pituitary response to a gonadotrophin-releasing hormone (GnRH) antagonist in monkeys: tonic follicle-stimulating hormone/luteinizing hormone secretion versus acute GnRH challenge tests before, during and after treatment. *Fertility and Sterility* **48**, 480-485.

Clarke, I.J. (1976). The effect of prenatal testosterone on reproductive patterns in ewes. Ph.D.thesis. University of Edinburgh.

Clarke, I.J. & Cummins, J.T. (1982). The temporal relationship between gonadotrophin releasing hormone (GnRH) and luteinizing hormone (LH) in ovariectomized ewes. *Endocrinology* **111**, 1737-1739.

Clarke, I.J. & Cummins, J.T. (1984). Direct pituitary effects of estrogen and progesterone on gonadotrophin secretion in the ovariectomized ewe. *Neuroendocrinology* **39**, 267-274.

Clarke, I.J. & Cummins, J.T. (1985). GnRH pulse frequency determines LH pulse amplitude by altering the amount of releasable LH in the pituitary glands of ewes. *Journal of Reproduction and Fertility* **73**, 425-431.

Clarke, I.J., Fraser, H.M. & McNeilly, A.S. (1978). Active immunization of ewes against luteinizing hormone releasing hormone and its effects on ovulation and gonadotrophin, prolactin and ovarian steroid secretion. *Journal of Endocrinology* **78**, 39-47.

Clayton, R.N. (1987). Gonadotrophin releasing hormone: from physiology to pharmacology. *Clinical Endocrinology* **26**, 361-384.

Clement, P.B. (1987). Histology of the ovary. *The American Journal of Surgical Pathologists* **11**(4), 277-303.

Collins, R.L., Sopelak, V.M., Williams, R.F. & Hodgen, G.D. (1986). Prevention of gonadotrophin-releasing hormone antagonist induced luteal regression by concurrent exogenous pulsatile gonadotrophin administration in monkeys. *Fertility and Sterility* **46**, 945-953.

Conn, P.M., Huckle, W.R., Andrews, W.V. & McArdle, C.A. (1987). The molecular mechanism of action of gonadotrophin releasing hormone (GnRH) in the pituitary. *Recent Progress in Hormone Research* **43**: 29-67.

Corner, G.W. (1956). The histological dating of the human corpus luteum of menstruation. *American Journal of Anatomy* **98**, 377-401.

Corrie, J.E.T., Ratcliffe, W.A. & Macpherson, J.S. (1982) The provision of ^{125}I-labelled tracers for radioimmunoassay of haptens: a general approach? *Journal of Immunological Methods* **51**, 159-166

Crisp, T.M., Dessouky, D.A. & Denys, F.R. (1970). The fine structure of the human corpus luteum of early pregnancy and during the progestational phase of the menstrual cycle. *American Journal of Anatomy* **127**, 37-70.

Crowder, M.E. & Nett, T.M. (1984). Pituitary content of gonadotrophins and receptors for gonadotrophin-releasing hormone (GnRH) and hypothalamic content of GnRH during the peri-ovulatory period of the ewe. *Endocrinology* **114**, 234-239.

Crowley, W.F.Jr., Filicori, M., Spratt, D.I. & Santoro, N.F. (1985). The physiology of gonadotrophin-releasing hormone (GnRH) secretion in men and women. *Recent Progress in Hormone Research* **41**, 473-531.

Croze, F. & Franchimont, P. (1984). Biological determination of inhibin in rat ovarian cell culture medium. *Journal of Reproduction and Fertility* **72**, 237-248.

Cuevas, P., Ying, S.Y., Ling, N., Ueno, N., Esch, F. & Guillemin, R. (1987). Immunohistochemical detection of inhibin in the gonad. *Biochemical and Biophysical Research Communications* **142** (1), 23-30.

Danforth, D.R., Sinosich, M.J., Anderson, T.L., Yan Cheng, C., Bardin, C.W. & Hodgen G.D. (1987) Identification of gonadotropin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. *Biology of Reproduction* **37**, 1075-1082.

Danforth, D.R., Williams, R.F., Hsiu, J.G., Roh, S.I., Hahn, D., McGuire, J.L. & Hodgen, G.D. (1990). Intermittent GnRH antagonist plus progestin contraception conserving tonic ovarian estrogen secretion and reducing progestin exposure. *Contraception* **41**(6), 623-631.

Davis, J.S., Alila, H.W., West, L.A., Corradino, R.A. & Hansel, W. (1988a). Acute effects of prostaglandin $F_{2\alpha}$ on inositol phospholipid hydrolysis in the large and small luteal cells of the bovine corpus luteum. *Molecular and Cellular Endocrinology* **58** (1), 43-50.

Davis, J.S., Weakland, L.L., Farese, R.V. & West, L.A. (1987a). Luteinizing hormone increases inositol triphosphate and cytosolic free Ca^{2+} in isolated bovine luteal cells. *Journal of Biological Chemistry* **262**, 8515-8521.

Davis, J.S., West, L.A., Weakland, L.L. & Farese, R.V. (1986a). Human chorionic gonadotrophin activates the inositol 1,4,5-triphosphate-Ca^{2+} intracellular signalling system in bovine luteal cells. *FEBS Letters* **208**, 287-291.

Davis, S.R., Burger, H.G., Robertson, D.M., Farnworth, P.G., Carson, R.S. & Krozowski, Z. (1988b). Pregnant mare's serum gonadotrophin stimulates inhibin subunit gene expression in the immature rat ovary: dose response characteristics and relationships to serum gonadotrophins, inhibin and ovarian steroid content. *Endocrinology* **123,** 2399-2407

Davis, S.R., Dench, F., Nikolaidis, I., Clementz, J.A., Forage, R.G., Krozowski, Z. & Burger, H.G. (1986b). Inhibin α-subunit gene is stimulated by pregnant mare serum gonadotrophin. *Biochemical and Biophysical Research Communications* **138,** 1191-1195.

Davis, S.R., Krozowski, Z., McLachlan, R.I. & Burger, H.G. (1987b). Inhibin gene expression in the human corpus luteum. *Journal of Endocrinology* **115**, R21-R23.

Dawood, M.Y. & Khan-Dawood, F.S. (1986). Human ovarian oxytocin: its source and relationship to steroid hormones. *American Journal of Obstetrics and Gynaecology* **154,** 756-763.

Dawood, M.Y., Raghaven, K.S., & Pociask, C. (1978). Radioimmunoassay of oxytocin. *Journal of Endocrinology* **76,** 261-270.

Deane, H.W., Lobel, B.L. & Romney, S.L. (1962). Enzymatic histochemistry of normal human ovaries of the menstrual cycle, pregnancy and the early puerperium. *American Journal of Obstetrics and Gynaecology* **83,** 281-294.

de Jong, F.H. & Sharpe, R.M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. *Nature* **263,** 71-72.

de Jong, F.H., Smith, S.D. & van der Molen, H.J. (1979). Bioassay of inhibin-like activity using pituitary cells *in vitro*. *Journal of Endocrinology* **80,** 91-102.

de Kretser, D.M. & Robertson, D.M. (1989). The isolation and physiology of inhibin and related proteins. *Biology of Reproduction* **40,** 33-47.

Dennefors, B.L., Sjogren, A. & Hamberger, L. (1982). Progesterone and adenosine 3', 5'-monophophate formation by isolated human corpora lutea of different ages: influence of human chorionic gonadotrophin and prostaglandins. *Journal of Clinical Endocrinology and Metabolism* **55**, 102-107.

De Paolo, L.V., Hirshfield, A.N., Anderson, L.D., Barraclough, C.A. & Channing, C.P. (1979). Suppression of pituitary secretion of follicle-stimulating hormone by porcine follicular fluid during pro-oestrus and oestrus in the rat: effects on gonadotrophin and steroid secretion, follicular development and ovulation during the following cycle. *Journal of Endocrinology* **83**, 355-368.

Depp, R., Cox, D.W., Pion, R.J., Conrad, S.H., & Heinrichs, W.L. (1973). Inhibition of the pregnenolone Δ^{5-4}-3β-hydroxysteroid dehydrogenase- Δ^{5-4}isomerase systems of human placenta and corpus luteum of pregnancy. *Gynecological Investigation* **4**, 106-120.

Diekman, M.A., O'Callahan, P.L., Nett, T.M. & Niswender, G.D. (1978). Validation of methods and quantification of luteal receptors for LH throughout the oestrous cycle and early pregnancy in ewes. *Biology of Reproduction* **19**, 999-1009.

Djahanbakhch, O., McNeilly, A.S., Hobson, B.M. & Templeton, A.A. (1981). A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. *British Journal of Obstetrics and Gynaecology* **88**, 1016-1020.

Dufau, M.L. & Catt, K.J. (1978). Gonadotrophin receptors and regulation of steroidogenesis in the testis and ovary. *Vitamins and Hormones* **36**, 462-592.

Eddie, L.W., Baker, H.W.G., Higginson, R.E. & Hudson, B. (1979). A bioassay for inhibin using pituitary cells in culture. *Journal of Endocrinology* **81**, 49-60.

Ellinwood, W.E., Norman, R.L.& Spies, H.G. (1984). Changing frequency of pulsatile LH and progesterone secretion during the luteal phase of the menstrual cycle of rhesus monkeys. *Biology of Reproduction* **31**, 714-722.

Ellinwood, W.E. & Resko, J.A. (1983). Effect of inhibition of oestrogen synthesis during the luteal phase on function of the corpus luteum in rhesus monkeys. *Biology of Reproduction* **28**, 636-644.

Elvidge, H. & Roper, C. (1977) Year to year breeding of primates (*Macaca mulatta.*) *Journal of the Institute of Animal Technicians* **28**, 37-45.

Erickson, L.W. & Hsueh, A.J.W. (1978). Secretion of "inhibin" by rat granulosa cells *in vitro*. *Endocrinology* **103**, 1960-1961.

Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N. & Guillemin, R. (1987). Structural characterization of follistatin: a novel follicle stimulating hormone release inhibiting polypeptide from the gonad. *Molecular Endocrinology* **1**, 849-855.

Eto., Y., Tsuji., T., Takezawa, M., Takano, S., Yokogawa, Y.& Shibai, H. (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochemical and Biophysical Research Communications* **42**, 1095-1103.

Eyster, K.M., & Stouffer, R.L. (1985). Adenylate cyclase in the corpus luteum of the rhesus monkey. II. Sensitivity to nucleotides, gonadotropins, catecholamines and nonhormonal activators. *Endocrinology* **116**, 1552-1558.

Feinberg, R. & Cohen, R.B. (1965). A comparative histochemical study of the ovarian stromal lipid band, stromal theca cell, and normal ovarian follicular apparatus. *American Journal of Obstetrics and Gynaecology* **92**, 958-969.

Ferin, M., Carmel, P.W., Zimmerman, E.A., Warren, M., Perez, R. & Vande Wiele, R.I. (1974). Location of intrahypothalamic estrogen-responsive sites influencing LH secretion in the female rhesus monkey. *Endocrinology* **95**, 1059-1068.

Ferin, M., Van Vugt, D. & Wardlaw, S. (1984). The hypothalamic control of the menstrual cycle and the role of endogenous opioids. *Recent Progress in Hormone Research* **40**, 441-480.

Filicori, M., Butler, J.P. & Crowley, W.F.Jr. (1984). Neuroendocrine regulation of the corpus luteum in the human. *Journal of Clinical Investigation* **73,** 1638-1647.

Findlay, J.K., Clarke, I.J. & Robertson, D.M. (1990a). Inhibin concentrations in ovarian and jugular venous plasma and the relationship of inhibin with FSH and LH during the ovine oestrous cycle. *Endocrinology* **126**, 528-535.

Findlay, J.K., Doughton, B., Robertson, D.M. & Forage, R.G. (1989a). Effects of immunization against recombinant bovine inhibin α-subunit on circulating concentrations of gonadotrophins in ewes. *Journal of Endocrinology* **120**, 59-65.

Findlay, J.K., Sai, X. & Shukovski, L. (1990b). Role of inhibin-related peptides as intragonadal regulators. *Reproduction, Fertility and Development* **2**, 205-218.

Findlay, J.K., Tsonis, C.G., Doughton, B. & Forage, R.G. (1989b). Immunization against the amino-terminal peptide (α_n) subunit of inhibin impairs fertility in sheep. *Endocrinology* **124**, 3122-3124.

Fingscheidt, U., Weinbauer, G.F., Robertson, D.M., de Kretser, D.M. & Nieschlag, E. (1989). Radioimmunoassay of inhibin in the serum of male monkeys. *Journal of Endocrinology* **122**, 477-483.

Fisch, B., Margare, R.A., Winston, R.M.L. & Hillier, S.G. (1989). Cellular basis of luteal steroidogenesis in the human ovary. *Journal of Reproduction and Fertility* **122**, 303-311.

Fitz, T.A., Hoyer, P.B. & Niswender, G.D. (1984a). Interactions of prostaglandins with subpopulations of ovine luteal cells. I. Stimulatory effects of prostaglandins E_1, E_2 and I_2. *Prostaglandins* **28**, 119-126.

Fitz, T.A., Mayan, M.H., Sawyer, H.R. & Niswender, G.D. (1982). Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biology of Reproduction* **27**, 703-711.

Fitz, T.A., Mock, E.J., Mayan, M.H. & Niswender, G.D. (1984b) Interactions of prostaglandins with subpopulations of ovine luteal cells. II. Inhibitory effects of $PGF_{2\alpha}$ and protection by PGE_2. *Prostaglandins* **287**, 127-135.

Flint, A.P.F., Auletta, F.J. & Barker, P.J. (1988). Isolation and sequence determination of a peptide detected by oxytocin radioimmunoassay in human corpus luteum. *Society for the Study of Reproduction* Abstract 90.

Flint, A.P.F. & Sheldrick, E.L. (1982). Ovarian secretion of oxytocin is stimulated by prostaglandin. *Nature* **297**, 587-588.

Flint, A.P.F. & Sheldrick, E.L. (1986). Ovarian oxytocin and the maternal recognition of pregnancy. *Journal of Reproduction and Fertility* **76**, 831-839.

Folkers, K., Bowers, C., Tang, P.F.L., Feng, D.M., Okamoto, T., Zhang, Y. & Ljungqvist, A. (1987). Specificity of design to achieve antagonists of LHRH of increasing effectiveness in therapeutic activity, In: *LHRH and its Analogs: Contraceptive and Therapeutic Applications. Part 2.* , pp. 17-24, Eds. B.H.Vickery and J.J.Nestor, Jr. M.T.P.Press, Lancaster.

Forage, R.G., Brown, R.W., Oliver, K.J., Atrache, B.T., Devine, P.L., Hudson, G.C., Goss, N.H., Bertram, K.C., Tolstoshev, P., Robertson, D.M., de Kretser, D.M., Doughton, B., Burger, H.G. & Findlay, J.K. (1987). Immunization against an inhibin subunit produced by recombinant DNA techniques results in increased ovulation rate in sheep. *Journal of Endocrinology* **114**, R1-R4.

Forage, R.G., Ring, J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T.W., Findlay, J.K., Wettenhall, R.E.H., Burger, H.G. & de Kretser, D.M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proceedings of the National Academy of Sciences* **83**, 3091-3095.

Fowler, P.A., Messinis, I.E. & Templeton, A.A. (1989) Gonadotrophin surge-attenuating factor (GnSAF) activity in human follicular fluid is different from inhibin. *Journal of Reproduction and Fertility*, Abstract Series No. 4, 30,abstract.

Franchimont, P., Hazee-Hagelstein, M.T., Charlet-Renard, Ch., & Jasper, J.N. (1986). The effect of mouse epidermal growth factor on DNA and protein synthesis progesterone and inhibin production by bovine granulosa cells in culture. *Acta Endocrinologica* **111**, 122-127.

Fraser, H.M. (1986). LHRH immunoneutralization-basic studies and prospects for practical application. In: *Immunological Approaches to Contraception and Promotion of Fertility*. Ed. G.P.Talwar. Plenum Press. New York and London.

Fraser, H.M. (1988a). LHRH analogues: their clinical physiology and delivery systems. *Bailliere's Clinical Obstetrics and Gynaecology* **2** (3), 639-658.

Fraser, H.M. (1988b). LHRH analogues: manipulation of pituitary ovarian function in macaques and potential clinical and contraceptive applications. In *Hormone Antagonists for Fertility Regulation* pp35-61. Eds. C.P.Puri, P.F.A.Van Look. Indian Society for the Study of Fertility.

Fraser, H.M., Abbott, M., Laird, N.C., McNeilly, A.S., Nestor, J.J. & Vickery, B.H. (1986). Effects of an LHRH antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (*Macaca arctoides*). *Journal of Endocrinology* **111**, 83-90.

Fraser, H.M. & Baird, D.T. (1987). Clinical applications of LHRH analogues. In *Bailliere's Clinical Obstetrics and Gynaecology* **1** (1), 43-70.

Fraser, H.M., Baird, D.T., McRae, G.I., Nestor, J.J. & Vickery, B.H. (1985). Suppression of luteal progesterone secretion in the stumptailed macaque by an antagonist analogue of luteinizing hormone-releasing hormone. *Journal of Endocrinology* **104**, R1-R4.

Fraser, H.M. & Lunn, S.F. (1989) Rapid inhibitory effects of an LHRH agonist implant on the oestrogen-induced LH surge and the induction of a defective luteal phase after an agonist-induced ovulation in the macaque. *Human Reproduction* **4**, 506-512.

Fraser, H.M. & McNeilly, A.S. (1982). Effects of immunoneutralization of luteinizing hormone releasing hormone on the oestrogen-induced luteinizing hormone and follicle stimulating hormone surges in the ewe. *Biology of Reproduction* **27**, 548-555.

Fraser, H.M. & McNeilly, A.S. (1983). Differential effects of LHRH immunoneutralization on LH and FSH secretion in the ewe. *Journal of Reproduction and Fertility* **69**, 569-577.

Fraser, H.M., McNeilly, A.S., Abbott, M. & Steiner, R.A. (1982). Effect of LHRH immunoneutralization on follicular development, the LH surge and luteal function in the stumptailed macaque monkey (*Macaca arctoides*). *Journal of Reproduction and Fertility* **76**, 299-309.

Fraser, H.M., Nestor, J.J.Jr. & Vickery, B.H. (1987a). Suppression of luteal function by a luteinizing hormone-releasing hormone antagonist during the early luteal phase in the stumptailed macaque monkey and the effects of subsequent administration of human chorionic gonadotrophin. *Endocrinology* **121**, 612-618.

Fraser, H.M., Robertson, D.M. & de Kretser, D.M. (1989). Inhibin concentrations in serum throughout the menstrual cycle of the macaque: suppression of inhibin during the luteal phase after treatment with an LHRH antagonist. *Journal of Endocrinology* **121**, R9-R12.

Fraser, H.M., Sandow, J., Cowen, G.M., Lumsden, M.A., Haining, R. & Smith, S.K. (1990). Long-term suppression of ovarian function by a luteinizing-hormone releasing hormone agonist implant in patients with endometriosis. *Fertility and Sterility* **53**, 61-68.

Fraser, H.M., Sandow, J., Seidel, H. & von Rechenberg, W. (1987b) An implant of a gonadotropin releasing hormone agonist (buserelin) which suppresses ovarian function in the macaque for 3-5 months. *Acta Endocrinologica* **115**, 521-527.

Fraser, I.S., Baird, D.T., Hobson, B.M., Michie, E.A. & Hunter, W. (1973). Cyclical ovarian function in women with congenital absence of the uterus and vagina. *Journal of Clinical Endocrinology and Metabolism* **36**, 634-637.

Fukada, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1986). Isolation of bovine follicular fluid of about 32 kDa. *Molecular and Cellular Endocrinology* **44**, 55-60.

Fuchs, A.R. (1988). Oxytocin and ovarian function. *Journal of Reproduction and Fertility* (Suppl.) **36**, 39-47.

Gillim, S.W., Christensen, A.K.& McLennan, C.E. (1969). Fine structure of the human menstrual corpus luteum at its stage of maximum secretory activity. *American Journal of Anatomy* **126**, 409-428.

Glasier, A.F., Irvine, D.S., Wickings, E.J., Hillier, S.G. & Baird, D.T. (1989). A comparison of the effects on follicular development between clomiphene citrate, its two separate isomers and spontaneous cycles. *Human Reproduction* **4** (3), 252-256.

Glasier, A., Thatcher, S.S., Wickings, E.J., Hillier, S.G. & Baird, D.T. (1988). Superovulation with exogenous gonadotropins does not inhibit the luteinizing hormone surge. *Fertility and Sterility* **49**, 81-85.

Goding, J.R. (1974). The demonstration that PGF is the uterine luteolysin in the ewe. *Journal of Reproduction and Fertility* **38**, 261-271.

Goodman, R.L. & Karsch, F.J. (1981). Control of seasonal breeding in the ewe: importance of changes in response to sex-steroid feedback. In *Biological Clocks in Seasonal Reproductive Cycles*. pp 223-236. Eds. B.K.Follett, D.E.Follett. Bristol: John Wright and Sons Ltd.

Gore, B.Z., Caldwell, B.V. & Speroff, L. (1973). Oestrogen-induced human luteolysis. *Journal of Clinical Endocrinology and Metabolism* **36**, 615-617.

Gospodarowicz, D. (1973). Properties of the luteinizing hormone receptor of the isolated bovine corpus luteum membrane. *Journal of Biological Chemistry* **248**, 5042-5049.

Guldenaar, S.E.F. (1984). Immunocytochemical evidence for the presence of oxytocin and neurophysin in the large cells of the bovine corpus luteum. *Cell and Tissue Research* **237**, 349-352.

Gulyas, B.J., Stouffer, R.L. & Hodgen, G.D. (1979). Progesterone synthesis and fine structure of dissociated monkey (*Macaca mulatta*) luteal cells maintained in culture. *Biology of Reproduction* **20**, 779-792.

Hahlin, M., Bennegard, B. & Dennefors, B. (1986). Human luteolysis-interaction between hCG and oestradiol-17β in an *in vitro* model. *Human Reproduction* **1**, 75-79.

Hall, P.F. (1985). Trophic stimulation of steroidogenesis: In search of the elusive trigger. *Recent Progress in Hormone Research* **82**, 559-568.

Halme, J., Ikonen, M., Rutanen, E.M. & Seppala, M. (1978). Gonadotrophin receptors of human corpus luteum during the menstrual cycle and pregnancy. *American Journal of Obstetrics and Gynaecology* **131**, 728-734.

Hamada, T., Watanabe, G., Kokuho, T., Taya, K., Sasamoto, S., Hasegawa, Y., Miyamoto, K. & Igarashi, M. (1989). Radioimmunoassay of inhibin in various mammals. *Journal of Endocrinology* **122**, 697-704.

Hamberger, L., Dennefors, B., Khan, I. & Sjogren, A. (1979). Cyclic AMP formation of isolated human corpora lutea in response to hCG-interference by $PGF_{2\alpha}$. *Prostaglandins* **17**, 615-621.

Hammerstein, J., Rice, B.F. & Savard, K. (1964). Steroid hormone formation in the human ovary: I. Identification of steroids formed in vitro from acetate-1-^{14}C in the corpus luteum. *Journal of Clinical Endocrinology and Metabolism* **24**, 597-605.

Hansel, W. & Dowd, J.P. (1986). New concepts of the control of corpus luteum function. *Journal of Reproduction and Fertility* **78**, 755-768.

Harding, R.D., Hulme, M.J., Lunn,S.F., Henderson, C. & Aitken, R.J. (1982). Plasma progesterone levels throughout the ovarian cycle of the common marmoset *(Callithrix jacchus). Journal of Medical Primatology* **11**, 43-51

Hardt, W. & Schmidt-Gollwitzer, M. (1984). Sustained gonadal suppression in fertile women with the LHRH agonist buserelin. *Clinical Endocrinology* **19**, 613-617.

Harlow, C.R., Gems, S., Hodges, J.K. & Hearn, J.P. (1983) The relationship between plasma progesterone and the timing of ovulation and early embryonic development in the marmoset monkey, *Callithrix jacchus. Journal of Zoology,* **201**, 265-278

Harlow, C.R., Hearn, J.P. & Hodges, J.K. (1984) Ovulation in the marmoset monkey: endocrinology, prediction and detection. *Journal of Endocrinology* **103**, 17-24

Hasegawa, Y., Miyamoto, K., Igarashi, M., Yanaka, T., Sasaki, K. & Iwamura, S. (1987). Changes in serum concentrations of inhibin during the oestrous cycle of the rat, pig and cow. *In 'Inhibin: Non-steroidal regulation and follicle stimulating hormone secretion.'* vol.42, 149-162. Eds. H.G.Burger, D.M.de Kretser, J.K.Findlay and M. Igarashi. Serono Symposium, Tokyo.Raven Press

Hasegawa, Y., Miyamoto, K., Iwamura, S. & Igarashi, M. (1988). Changes in serum concentrations of inhibin in cyclic pigs. *Journal of Endocrinology* **118**, 211-219.

Healy, D.L., Lawson, S.R., Abbott, M., Baird, D.T. & Fraser, H.M. (1986). Towards removing uterine fibroids without surgery: subcutaneous infusion of a luteinizing hormone-releasing hormone agonist commencing in the luteal phase. *Journal of Clinical Endocrinology and Metabolism* **63**, 619-625.

Hearn, J.P. (1978) The endocrinology of reproduction in the common marmoset, *Callithrix jacchus* . In: *The Biology and Conservation of Marmosets* pp.163-171 Ed. D.G.Kleiman.Washington: Smithsonian Press.

Hearn, J.P. (1983) The common marmoset *(Callithrix jacchus)* . In: *Reproduction in New World Primates* pp 181-215. Ed. J.P.Hearn. Lancaster: MTP Press

Hearn, J.P., Abbott, D.H., Chambers, P.C., Hodges, J.K. & Lunn, S.F.(1978) Use of the common marmoset, *Callithrix jaccus*, in reproductive research. In: *Primates in Medicine* Vol., 10, 40-49. Eds. E.I.Goldsmith & J.Moor-Jankowski.Basel: Karger

Hedger, M.P., Drummond, A.E., Robertson, D.M., Risbridger, G.P.& de Kretser, D.M. (1989). Inhibin and activin regulate ^{3}H-thymidine uptake by rat thymocytes and 3T3 cells *in vitro. Molecular and Cellular Endocrinology* **61**, 133-138.

Henderson, K.M. & Franchimont, P. (1981). Regulation of inhibin production by bovine ovarian cells *in vitro. Journal of Reproduction and Fertility* **63**, 431-442.

Henderson, K.M. & McNatty, K.P. (1977). A possible inter-relationship between gonadotrophin stimulation and prostaglandin $F_{2\alpha}$ inhibition of steroidogenesis by granulosa-luteal cells *in vitro*. *Journal of Endocrinology* **73**, 71-78

Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M., Sander, H.J. & de Jong, F.H. (1982). Estimation of inhibin-like activity in spent medium from rat ovarian granulosa cells during long term culture. *Molecular and Cellular Endocrinology* **27**, 277-290.

Hild-Petito, S.A., Shiigi, S.M. & Stouffer, R.L. (1989). Isolation and characterization of cell populations from the monkey corpus luteum of the menstrual cycle. *Biology of Reproduction* **40**, 1075-1085.

Hild-Petito, S., Stouffer, R.L. & Brenner, R.M. (1988). Immunocytochemical localization of estradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology* **123**, 2896-2905.

Hillier, S.G. & Read, G.F. (1975). Radioimmunoassay for plasma norethisterone and testosterone using anti-sera raised against C-11 conjugated haptens and radio-iodinated ligands. *Journal of Endocrinology* **67**, 5P-6P

Hillier, S.G., Wickings, E.J., Illingworth, P.J., Yong, E.L., Reichert, L.E., Baird, D.T. & McNeilly, A.S. (1991a). Control of inhibin production by human granulosa cells. Submitted for publication. *Clinical Endocrinology.*

Hillier, S.G., Wickings, E.J., Saunders, P.T.K., Dixson, A.F., Shimasaki, S., Swanston, I.A., Reichert, L.E.Jr. & McNeilly, A.S. (1989). Control of inhibin production by primate granulosa cells. *Journal of Endocrinology* **123**, 65-73.

Hillier, S.G., Yong, E.L., Illingworth, P.J., Baird, D.T., Schwall, R.H. & Mason, A.J. (1991b). Inhibitory effect of recombinant human activin A on androgen synthesis in cultured human thecal cells. *Journal of Clinical Endocrinology and Metabolism.* in press.

Hobson, B.M., Hearn, J.P., Lunn, S.F. & Flockhart, J.P. (1977). Urinary excretion of biologically active chorionic gonadotrophin by the pregnant marmoset *(Callithrix jacchus). Folia Primatologica* **28**, 251-258

Hodges J.K., Green, P.G., Cottingham, P.G., Sauer, M.J., Edwards, C. & Lightman S.L. (1988). Induction of luteal regression in the marmoset monkey (*Callithrix jacchus*) by a gonadotrophin-releasing hormone antagonist and the effects on subsequent follicular development. *Journal of Reproduction and Fertility* **82**, 743-752.

Hodges, J.K., Henderson, C. & Hearn, J.P. (1983) Relationship between ovarian and placental steroid production during early pregnancy in the marmoset monkey. *Journal of Reproduction and Fertility* **69**, 613-621

Horton, E.W. & Poyser, N.L. (1976). Uterine luteolytic hormone: a physiological role for prostaglandin $F_{2\alpha}$. *Physiological Reviews* **56**, 595-

Hoyer, P.B., Fitz, T.A. & Niswender, G.D. (1984). Hormone-dependent activation of adenylate cyclase in large steroidogenic ovine luteal cells does not result in increased progesterone secretion. *Endocrinology* **114**, 604-608.

Hoyer, P.B. & Niswender, G.D. (1986). Adenosine 3', 5'-monophosphate binding capacity in small and large ovine luteal cells. *Endocrinology* **119**, 1822-1829.

Hsu, S.M., Raine, L & Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry* **29**, 577-580.

Hsueh, A.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. & Vale, W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proceeding of the National Academy of Sciences USA* **84**, 5082-5086.

Hunter, M.G. & Baker, T.G. (1981). Effect of hCG, cAMP and FSH on steroidogenesis by human corpora lutea in vitro. *Journal of Reproduction and Fertility* **63**, 285-288.

Hunter, W.M., Nors, P.W. & Rutherford, F.J. (1975). Preparations and behaviour of ^{125}I labelled radioligand for phenolic and neutral steroids. In *Steroid Immunoassay*. Proceeedings of the Vth Tenovus Workshop. Meron. pp 141-152. Eds. Cameron, E.H.D., Hillier, S.G. & Griffiths, J.K. Alpha Omega Alpha Publishing Ltd, Cardiff.

Hutchison, J.S., Nelson, P.B. & Zeleznik, A.J. (1986). Effects of different gonadotrophin pulse frequencies on corpus luteum function during the menstrual cycle of rhesus monkey. *Endocrinology* **119**, 1964-1971.

Hutchison, J.S. & Zeleznik, A.J. (1984). The rhesus monkey corpus luteum is dependent on pituitary gonadotrophin secretion throughout the luteal phase of the menstrual cycle. *Endocrinology* **115**, 1780-1786.

Hutchison, J.S. & Zeleznik, A.J. (1985). The corpus luteum of the primate menstrual cycle is capable of recovering from a transient withdrawal of pituitary gonadotrophin support. *Endocrinology* **117**, 1043-1049.

Ivell, R., Furuya, K., Brackmann, B., Dawood, Y. & Khan-Dawood, F. (1990). Expression of the oxytocin and vasopressin genes in human and baboon gonadal tissues. *Endocrinology* **127**, 2990-2996.

Jackson, G.L. (1975). Blockage of estrogen-induced release of luteinizing hormone by reserpine and potentiation of synthetic gonadotrophin-releasing hormone-induced release of luteinizing hormone by estrogen in the ovariectomized ewe. *Endocrinology* **97**, 1300-1307.

Johnson, M.S., Ottobre, A.C. & Ottobre, J.S. (1988). Prostaglandin production by corpora lutea of rhesus monkeys: characterization of incubation conditions and examination of putative regulators. *Biology of Reproduction* **39**, 839-846.

Karsch, F.J. (1987). Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. *Annual Review of Physiology* **49**, 365-382.

Karsch, F.J., Dierschke, D.J., Weick, R.F., Yamaji, T., Hotchkiss, J. & Knobil, E. (1973a). Positive and negative feedback control by oestrogen of luteinizing hormone secretion in the rhesus monkey. *Endocrinology* 92, 799-804.

Karsch, F.J., Krey, L.C., Weick, R.F., Dierschke, D.J. & Knobil, E. (1973b). Functional luteolysis in the rhesus monkey: role of oestrogens. *Endocrinology* **92**, 1148-1152.

Karsch, F.J. & Sutton, G.P. (1976). An intra-ovarian site for the luteolytic action of oestrogen in the rhesus monkey. *Endocrinology* **98**, 553-561.

Karsch, F.J., Weick, R.F., Butler, W.R., Dierschke, D.J., Krey, L.C., Weiss, G., Hotchkiss, J., Yamaji, T. & Knobil, E. (1973c). Induced LH surges in the rhesus monkey: Strength-duration characteristics of the oestrogen stimulus. *Endocrinology* **92**, 1740-1747.

Karsch, F.J., Weick, R.F., Hotchkiss, J., Dierschke, D.J. & Knobil, E. (1973d). An analysis of the negative feedback control of gonadotrophin secretion utilizing chronic implantation of ovarian steroids in ovariectomized rhesus monkeys. *Endocrinology* **93**, 478-486.

Kaufman, M., Kesner, S.J., Wilson, R.C. & Knobil, E. (1985). Electrophysiological manifestation of luteinizing hormone-releasing hormone pulse generator activity in the Rhesus monkey: influence of α-adrenergic and dopaminergic blocking agents. *Endocrinology* **116**, 1327-1333.

Kawai, Y. & Clark, M.R. (1985). Phorbol ester regulation of rat granulosa cell prostaglandin and progesterone accumulation. *Endocrinology* **116**, 2320-2326.

Kenigsberg, D. & Hodgen, G.D. (1986). Ovulation inhibition by administration of weekly gonadotrophin-releasing hormone antagonist. *Journal of Clinical Endocrinology and Metabolism* **62**, 734-736.

Khan-Dawood, F.S. (1986). Localization of oxytocin and neurophysin in baboon (*Papio anubis*) corpus luteum by immunocytochemistry. *Acta Endocrinologica* **113**, 570-575.

Khan-Dawood, F.S. (1987a). Oxytocin in baboon (*Papio anubis*) corpus luteum. *Biology of Reproduction* **37**, 659-664.

Khan-Dawood, F.S. (1987b). Immunocytochemical localization of oxytocin and neurophysin in human corpus luteum. *American Journal of Anatomy* **179**, 18-24.

Khan-Dawood, F.S. & Dawood, M.Y. (1983). Human ovaries contain immunoreactive oxytocin. *Journal of Clinical Endocrinology and Metabolism* **57**, 1129-1132.

Khan-Dawood, F.S., Goldsmith, L.T., Weiss, G. & Dawood, M.Y. (1989). Human corpus luteum secretion of relaxin, oxytocin and progesterone. *Journal of Clinical Endocrinology and Metabolism* **68**, 627-631.

Khan-Dawood, F.S., Marut, E.L. & Dawood, M.Y. (1983). Oxytocin in the corpus luteum of the cynomolgus monkey (*Macaca fascicularis*). *Endocrinology* **115**, 570-574.

Kirton, K.T., Pharriss, B.B.& Forbes, A.D. (1970). Luteolytic effects of $PGF_{2\alpha}$ in primates. *Proceedings of the Society for Experimental Biology and Medicine* **133**, 341-350

Kitaoka, M., Yamashita, N., Eto, Y., Shibai, H. & Ogata, E. (1987). Stimulation of FSH release by erythroid differentiation factor (EDF). *Biochemical and Biophysical Research Communications* **146**, 1382-1385.

Knight, P.G., Beard, A.J., Wrathall, J.H.M. & Castillo, R.J. (1989). Evidence that the bovine ovary secretes large amounts of monomeric inhibin α-subunit and its isolation from bovine follicular fluid. *Journal of Molecular Endocrinology* **2**, 189-200.

Knobil, E. (1974). On the control of gonadotrophin secretion in the rhesus monkey. *Recent Progress in Hormone Research* **30**, 1-46.

Knobil, E. (1980). The neuroendocrine control of the menstrual cycle. *Recent Progress in Hormone Research* **36**, 53-89.

Knobil, E. (1981). Patterns of hypophysiotropic signals and gonadotrophin secretion in the rhesus monkey. *Biology of Reproduction* **24**, 44-49.

Koch, Y., Chobsieng, P., Zor, U., Fridkin, M. & Lindner, H.R. (1973). Suppression of gonadotrophin secretion and prevention of ovulation in the rat by antiserum to synthetic gonadotrophin releasing hormone. *Biochemical and Biophysical Research Communications* **55**, 623-629.

Koos, R.D. & Hansel, W. (1981). The large and small cells of the bovine corpus luteum: ultrastructural and functional differences. In: *Dynamics of Ovarian Function.* pp197-202. Eds. N.B. Schwartz, M. Hunzicker-Dunn. Raven Press. New York.

Korda, A.R., Shutt, D.A., Smith, I.D., Shearman, R.P. & Lyneham, R.C. (1975). Assessment of possible luteolytic effect of intra-ovarian injection of prostaglandin $F_{2\alpha}$ in the human. *Prostaglandins* **9**, 443-449.

Kruip, T.A.M., Vullings, H.G.B., Schamms, D., Jonis, J. & Klarenbeck, A. (1985). Immunocytochemical demonstration of oxytocin in bovine ovarian tissues. *Acta Endocrinologica* **109**, 537-542.

Laherty, R.F., Rotten, D., Yamamoto, M. & Jaffe, R.B. (1985). Effects of oestradiol and prolactin on progesterone production by rhesus monkey luteal cells *in vitro*. *Acta Endocrinologica* **79**, 329-336.

Lalloz, M.R.A., Detta, A. & Clayton, R.N. (1988). Gonadotrophin-releasing hormone desensitization preferentially inhibits expression of the luteinizing hormone β subunit gene *in vivo*. *Endocrinology* **122**, 1689-1694.

Lappohn, R.E., Burger, H.G., Bouma, J., Bangah, M., Krans, M. & de Bruijn, H.W.A. (1989). Inhibin as a marker for granulosa-cell tumors. *The New England Journal of Medicine* **321**, 790-793.

Leal, J.A., Gordon, K., Williams, R.F., Danforth, D.R., Roh, S.I. & Hodgen, G.D. (1989). Probing studies on multiple dose effects of antide (NAL-LYS) GnRH antagonist in ovariectomized monkeys. *Contraception* **40**, 623-633.

Lee, V.W.K. (1983). PMSG-treated immature female rat- a model system for studying control of inhibin secretion. In *'Factors Regulating Ovarian Function.'* p. 157-161. Eds. G.S.Greenwald and P.F.Terranova. Raven Press: New York.

Lemon, M. & Loir, M. (1977). Steroid release *in vitro* by two cell types in the corpus luteum of the pregnant sow. *Journal of Endocrinology* **72**, 351-359.

Lemon, M. & Mauleon, P. (1982). Interaction between two luteal cell types in the corpus luteum of the sow in progesterone synthesis *in vitro*. *Journal of Reproduction and Fertility* **64**, 315-323.

Levine, J.E., Pau, K-Y, Ramirez, V.D. & Jackson, G.I. (1982). Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanaesthetized, ovariectomized sheep. *Endocrinology* **111**, 1449-1455.

Levine, J.E., Norman, R.L., Gleissman, P.M., Oyama, T.T., Bangsberg, D.R. & Spies, H.G. (1985). In vivo gonadotrophin-releasing hormone release in serum luteinizing hormone measurements in ovariectomized, estrogen-treated rhesus macaques. *Endocrinology* **117**, 711-721.

Leyendecker, G., Wardlaw, S. & Nocke, W. (1972). Experimental studies on the endocrine regulations during the periovulatory phase of the human menstrual cycle. *Acta Endocrinologica* **71**, 160-178.

Li, T.C., Rogers, A.W., Dockery, P., Lenton, E.A. & Cooke, I.D. (1988). A new method of histologic dating of human endometrium in the luteal phase. *Fertility and Sterility* **50**, 52-60.

Lincoln, G.A. & Fraser, H.M. (1979). Blockade of episodic secretion of luteinizing hormone in the ram by the administration of antibodies to luteinizing hormone releasing hormone. *Biology of Reproduction* **21**, 1239-1245.

Lincoln, G.A. & Fraser, H.M. (1987). Compensatory response of the luteinizing-hormone (LH)-releasing hormone (LHRH)/LH pulse generator after administration of a potent LHRH antagonist in the ram. *Endocrinology* **120**, 2245-2250

Lincoln, G.A. & Fraser, H.M. (1990). Negative feedback regulation of pulsatile LH secretion during treatment with an LHRH antagonist in rams. *Journal of Andrology* **11**, 287-292.

Ling, N., Ying, S.Y., Ueno, H., Esch, F., Denorary, L. & Guillemin, R. (1985). Isolation and partial characterization of a Mw 32,000 protein with inhibin activity from porcine follicular fluid. *Proceedings of the National Academy of Sciences U.S.A.* **82**, 7217-7221.

Ling, N., Ying, S.Y., Ueno, H., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986a). A homodimer of the β subunits of inhibin A stimulates the secretion of pituitary follicle-stimulating hormone. *Biochemical and Biophysical Research Communications* **138**, 1129-1137.

Ling, N., Ying, S.Y., Ueno, H., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986b). Pituitary FSH is released by a heterodimer of the β subunits of the two forms of inhibin. *Nature* **321**, 779-782.

Lipsett, M. B., Chrousos, G.P., Tomita, M., Brandon, D. D. & Loriaux, D.L. (1985). The defective glucocorticoid receptor in man and nonhuman primates. *Recent Progress in Hormone Research* **41**, 199-247

Littman, B.A. & Hodgen, G.D. (1984) Human menopausal gonadotropin stimulation in monkeys: blockade of the luteinizing hormone surge by a highly transient ovarian factor. *Fertility and Sterility* **41**, 440-447.

Lumsden, M.A., West, C.P. & Baird, D.T. (1989). Tamoxifen prolongs luteal phase in premenopausal women but has no effect of the size of uterine fibroids. *Clinical Endocrinology* 31, 335-344.

Macnaughton, M.C., Kader, A.S., Gaukroger, J. & Coutts, J.R.T. (1981). Steroid production by the human corpus luteum. In *Functional morphology of the human ovary.* pp137-153. Ed. J.R.T. Coutts :Lancaster: M.T.P.Press Ltd.

Mais, V., Kazer, R.R., Cetel, N.S., Rivier, J., Vale, W. & Yen, S.S.C. (1986). The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotrophin secretion in cycling women using a gonadotrophin-releasing hormone antagonist as a probe. *Journal of Clinical Endocrinology and Metabolism* **62**, 1250-1255.

Marrs, R.P., Lobo, R., Campeau, J.D., Nakamura, R.M., Brown, J., Ujita, E.L. & diZerega, G.S. (1984). Correlation of human follicular fluid inhibin activity with spontaneous and induced follicle maturation. *Journal of Clinical Endocrinology and Metabolism* **58**, 704-709.

Marshall, G.R., Aktar, F.B., Weinbauer, G.F. & Nieschlag, E. (1986). Gonadotrophin-releasing hormone (GnRH) overcomes GnRH antagonist-induced suppression of LH secretion in primates. *Journal of Endocrinology* **110**, 145-150.

Mason, A.J., Berkemeier, L.M., Schmelzer, C.H. & Schwall, R.H. (1989). Activin B- precursor sequences, genomic structure and *in vitro* activities. *Molecular Endocrinology* **3**(9), 1352-1358.

Mason, A.J., Hayflick, J.S., Esch, F., Ueno, H., Ying, S.Y., Guillemin, R., Niall, H. & Seeburg, P.H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. *Nature* **318**, 659-663.

Mason, A.J., Niall, H.D. & Seeburg, P.H. (1986). Structure of two human ovarian inhibins. *Biochemical and Biophysical Research Communications* **135**, 957-964.

Maruncic, M. & Casper, R.F. (1987). The effect of luteal phase estrogen antagonism on luteinizing hormone pulsatility and luteal function in women. *Journal of Clinical Endocrinology and Metabolism* **64**, 148-152.

Marut, E.L., Huang, S-C. & Hodgen, G.D. (1983). Distinguishing the steroidogenic roles of granulosa and theca cells of the dominant ovarian follicle and corpus luteum. *Journal of Clinical Endocrinology and Metabolism* **57**, 925-930.

Mayo, K.E., Cerelli, C.M., Spies, J., Rivier, J., Rosenfield, M.G., Evans, R.M. & Vale, W. (1986). Inhibin α-subunit cDNA from porcine ovary and human placenta. *Proceedings of the National Academy of Sciences U.S.A.* **83**, 5849-5853.

McCann, S.M., Samson, W.K. & Aguila, M.C., (1986). The role of brain peptides in the control of anterior pituitary hormone secretion. In *Neuroendocrine Molecular Biology*, pp101-112. Eds. Fink, G., Harmer, A.J., McKerns, K.W. New York: Plenum Press.

McCracken, J.A., Schramm, W., Barcikowski, B. & Wilson, L. (1981). The identification of prostaglandin $F_{2\alpha}$ as a uterine luteolytic hormone in the sheep and the endocrine control of its synthesis. *Acta Veterinaria Scandinavia (Suppl.)* **77**, 71-88.

McCullagh, D.R. (1932). Dual endocrine activity of the testes. *Science* **76**, 19-20.

McKay, D.G., Pinkerton, J.H.M., Hertig, A.T. & Danziger, S. (1961). The adult human ovary: a histochemical study. *Obstetrics and Gynaecology* **18**, 13-39.

McLachlan, R.I., Burger, H.G., Healy, D.L., de Kretser, D.M. & Robertson, D.M. (1987c). Circulating immunoreactive inhibin in the luteal phase and early gestation of women undergoing ovulation induction. *Fertility and Sterility* **48**, 1001-1005.

McLachlan, R.I., Cohen, N.L., Dahl, K.D., Bremner, W.J. & Soules, M.R. (1990). Serum inhibin levels during the periovulatory interval in normal women: relationships with sex steroids and gonadotrophin levels. *Clinical Endocrinology* **32**, 39-48.

McLachlan, R.I., Cohen, N.L., Vale, W.W., Rivier, J.E., Burger, H.G., Bremner, W.J. & Soules, M.R. (1989). The importance of luteinizing hormone in the control of inhibin and progesterone secretion in the human corpus luteum. *Journal of Clinical Endocrinology and Metabolism* **68**, 1078-1085.

McLachlan, R.I., Healy, D.L., Lutjen, P.J., Findlay, J.K., de Kretser, D.M. & Burger, H.G. (1987b). The maternal ovary is not the source of circulating inhibin levels during human pregnancy. *Clinical Endocrinology* **27**, 663-668.

McLachlan, R.I., Healy, D.L., Robertson, D.M., Burger, H.G. and de Kretser, D.M. (1986b). The human placenta: a novel source of inhibin. *Biochemical and Biophysical Research Communications* **140**, 485-490.

McLachlan, R.I., Robertson, D.M., Burger, H.G. & de Kretser, D.M. (1986a). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. *Molecular and Cellular Endocrinology* **46**, 175-185.

McLachlan, R.I., Robertson, D.M., de Kretser, D.M. & Burger, H.G. (1988) Advances in the physiology of inhibin and inhibin-related peptides. *Clinical Endocrinology* **29**, 77-114.

McLachlan, R.I., Robertson, D.M., Healy, D.L., de Kretser, D.M. & Burger, H.G. (1986c). Plasma inhibin levels during gonadotrophin-induced ovarian hyperstimulation for IVF: a new index of follicular maturation? *Lancet* **i**, 1233-1234.

McLachlan, R.I., Robertson, D.M., Healy, D.L., Burger, H.G. & de Kretser, D.M. (1987a). Circulating immunoreactive inhibin levels during the normal human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **65**, 954-961.

McNatty, K.P., Baird, D.T., Bolton, A., Chambers, P.S., Corker, C.S. & McLean, H. (1976). Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle. *Journal of Endocrinology* **71**, 77-85.

McNatty, K.P., Henderson, K.M. & Sawers, R.S. (1975). Effects of prostaglandin $F_{2\alpha}$ and E_2 on the production of progesterone by human granulosa cells in tissue culture. *Journal of Endocrinology* **67**, 231-240.

McNeilly, A.S. & Baird, D.T. (1989). Episodic secretion of inhibin into the ovarian vein during the follicular phase of the oestrous cycle in the ewe. *Journal of Endocrinology* **122**, 287-292.

McNeilly, A.S., Fraser, H.M. & Baird, D.T. (1984). Effect of immunoneutralization of luteinizing hormone releasing hormone on LH, FSH and ovarian steroid secretion in the preovulatory phase of the oestrus cycle in the ewe. *Journal of Endocrinology* **101**, 213-219.

McNeilly, A.S., Kerin, J., Swanston, I.A., Bramley, T.A. & Baird, D.T. (1980). Changes in the binding of human chorionic gonadotrophin/luteinizing hormone and prolactin to human corpora lutea during the menstrual cycle and early pregnancy. *Journal of Endocrinology* **87**, 315-325.

McNeilly, A.S., Swanston, I.A., Crow, W., Tsonis, C.G. & Baird, D.T. (1989). Changes in plasma concentrations of inhibin throughout the normal sheep oestrous cycle and after the infusion of FSH. *Journal of Endocrinology* **120**, 295-305.

McNeilly, A.S., Tsonis, C.G. & Baird, D.T. (1988). Inhibin. *Human Reproduction* **3** (1), 45-49.

Messinis, I.E. & Templeton, A.A. (1987) Endocrine and follicle characteristics of cycles with and without endogenous luteinizing hormone surges during superovulation induction with pulsatile follicle-stimulating hormone. *Human Reproduction* **2**, 11-16.

Messinis, I.E. & Templeton, A.A. (1989) Pituitary response to exogenous LHRH in superovulated women. *Journal of Reproduction and Fertility* **87**, 633-639.

Meunier, H., Cajander, S.B., Roberts, V.J., Sawchenko, P.E., Hsueh, A.J.W. & Vale, W. (1988a). Rapid changes in the expression of inhibin α-, βA- and βB-subunits in ovarian cell types during the rat oestrous cycle. *Molecular Endocrinology* **2**, 1352-1363.

Meunier, H., Rivier, C., Evans, R.M. & Vale, W. (1988b). Gonadal and extragonadal expression of inhibin α, βA and βB subunits in various tissues predicts diverse functions. *Proceedings of the National Academy of Sciences U.S.A.* **85**, 247-251.

Milne, J.A. (1983). Effects of oxytocin on the oestrus cycle of the ewe. *Australian Veterinary Journal* **39**, 51-52.

Miyamoto, K., Hasegawa, Y., Fukada, M. and Igarashi, M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bff) by using monoclonal antibodies to bff 32K inhibin. *Biochemical and Biophysical Research Communications* **136**, 1103-1109.

Miyamoto, K., Hasegawa, Y., Fukada, M., Nomura, M., Igarashi, M., Kangawa, M. & Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32 kDa. *Biochemical and Biophysical Research Communications* **129**, 396-403.

Molskness, T.A., Van de Voort, C.A. & Stouffer, R.L. (1987). Stimulatory and inhibitory effects of prostaglandins on the gonadotrophin-sensitive adenylate cyclase in the monkey corpus luteum. *Prostaglandins* **34**, 279-290.

Monroe, S.E., Jaffe, R.B. & Midgely, A.R.Jr. (1972). Regulation of human gonadotropins XII. Increase in serum gonadotropins in response to estradiol. *Journal of Clinical Endocrinology and Metabolism* **34**, 342-347.

Moore, H.D.M., Gems, S. & Hearn, J.P. (1985) Early implantation stages in the marmoset monkey *(Callithrix jacchus). American Journal of Anatomy* **172**, 265-278

Morris, M., Stevens, S.W. & Adams, M.R. (1980). Plasma oxytocin during pregnancy and lactation in the cynomolgus monkey (*Macaca fascicularis*). *Biology of Reproduction* **23**, 782-787.

Moss, G.E., Crowder, M.E. & Nett, T.M. (1981). GnRH-receptor and interaction.VI. Effect of progesterone and estradiol on hypophyseal receptors for GnRH and serum and hypophyseal concentrations of gonadotrophins in ovariectomized ewes. *Biology of Reproduction* **25**, 938-944.

Nakai, Y., Plant, T.M., Hess, D.L., Keogh, E.J. & Knobil, E. (1978). On the sites of the negative and positive feedback actions of estradiol in the control of gonadotrophin secretion in the rhesus monkey. *Endocrinology* **102**, 1008-1014.

Nakajima, S.T., McLachlan, R.I., Cohen, N.L., Clifton, D.K., Bremner, W.J. & Soules, M.R. (1990). The immunoreactive inhibin secretion pattern in the midluteal phase: relationships with luteinizing hormone and progesterone. *Clinical Endocrinology* **33**, 709-717.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. & Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.

Neill, J.D., Patton, J.M., Dailey, R.A., Tsou, R.C. & Tindall, G.T. (1977). Luteinizing hormone releasing hormone (LHRH) in pituitary stalk blood of rhesus monkeys: relationship to level of LH release. *Endocrinology* **101**, 430-434.

Nelson, W.W. & Greene, R.R. (1958). Some observations of the histology of the human ovary during pregnancy. *American Journal of Obstetrics and Gynaecology* **76**, 66-89.

Niswender, G.D., Monroe, S.E., Peckman, W.D., Midgley, A.R., Knobil, E. & Reichert, L.E. (1971). Radioimmunoassay of rhesus monkey luteinizing hormone (LH) with anti-ovine LH serum and ovine LH-^{131}I. *Endocrinology* **88**, 1327-1331.

Niswender, G.D., Reimers, T.J., Diekman, M.A. & Nett, T.M. (1976). Blood flow: a mediator of ovarian function. *Biology of Reproduction* **14**, 64-81.

Niswender, G.D., Schwall, R.H., Fitz, T.A., Farin, C. & Sawyer, H.R. (1985). Regulation of luteal function in domestic ruminants: new concepts. *Recent Progress in Hormone Research* **41**, 101-151.

Noguchi, K., Keeping, H.S., Winters, S.J., Oshima, H. & Troen, P. (1987). Identification of inhibin secreted by cynomolgus monkey Sertoli cell cultures. *Journal of Clinical Endocrinology and Metabolism* **64**, 783-788.

Norman, R.L., Lindstrom, S.A., Bangsberg, D., Ellinwood, W.E., Gleissman, P, & Spies, H.G. (1984). Pulsatile secretion of luteinizing hormone during the menstrual cycle of rhesus macaques. *Endocrinology* 115, 261-266.

Noyes, R.W., Hertig, A.T. & Rock, J. (1950) Dating the endometrial biopsy. *Fertility and Sterility* **1**, 3-25.

O, W.-S., Robertson, D.M. & de Kretser, D.M. (1989). Inhibin as an oocyte meiotic inhibitor. *Molecular and Cellular Endocrinology* **62**, 307-311.

Ohara, A., Mori, T., Taii, S., Ban, C. & Narimoto, K. (1987). Functional differentiation in steroidogenesis of two cell types of luteal cells isolated from mature human corpora lutea of menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **65**, 1192-1200.

Olive, D.L., Schultz, N., Riehl, R.M., Groff, T.R. & Schenken, R.S. (1990). Effects of tamoxifen on corpus luteum function and luteal phase length in cynomolgus monkeys. *Fertility and Sterility* **54** (2), 333-338.

Patwardhan, V.V. & Lanthier, A. (1980). Concentration of prostaglandins, PGE, PGF, oestrone, oestradiol and progesterone in human corpora lutea. *Prostaglandins* **20**, 963-969.

Patwardhan, V.V. & Lanthier, A. (1984). Effect of prostaglandin $F_{2\alpha}$ on the hCG-stimulated progesterone production from human corpora lutea. *Prostaglandins* **27**, 465-473.

Pfaff, D.W., Gerlach, J.L., McEwen, B.S., Ferin, M., Carmel, P. & Zimmerman, E.A. (1976). Autoradiographic localization of hormone-concentrating cells in the brain of the female rhesus monkey. *Journal of Comparative Neurology* **170**, 279-294.

Phillips, H.S., Nikolics, K., Branton, D. & Seeburg, P.H. (1985). Immunocytochemical localization in rat brain of a prolactin release-inhibiting sequence of gonadotrophin-releasing hormone prohormone. *Nature* **316**, 542-545.

Plant, T.M., Nakai, Y., Belchetz, P., Keogh, E.J. & Knobil, E. (1978). The sites of action of estradiol and phentolamine in the inhibition of the pulsatile, circhoral discharges of LH in the rhesus monkey (*Macaca mulatta*). *Endocrinology* **102**, 1015-1018.

Plevrakis, I., Clamagirand, C. & Pontonnier, G. (1990). Oxytocin biosynthesis in serum-free cultures of human granulosa cells. *Journal of Endocrinology* **124**, R5-R8.

Pohl, C.R. & Knobil, E. (1982). The role of the central nervous system in the control of ovarian function in higher primates. *Annual Review of Physiology* **44**, 583-593.

Powell, W.S., Hammarstrom, S., Samuelsson, B. & Sjoberg, B. (1974). Prostaglandin $F_{2\alpha}$ receptor in human corpora lutea. *Lancet* **1**, 1120.

Rao, Ch.V., Griffin, L.P. & Carmen, Jr.F.R. (1977a). Gonadotrophin receptors in human corpora lutea of the menstrual cycle and pregnancy. *American Journal of Obsetetrics and Gynaecology* **128**, 146-153.

Rao, Ch.V., Griffin, L.P. & Carmen, Jr.F.R. (1977b). Prostaglandin $F_{2\alpha}$ binding sites in human corpora lutea. *Journal of Clinical Endocrinology and Metabolism* **44**, 1032-1037.

Ravindranath, N. & Moudgal, N.R. (1987). Use of tamoxifen, an antioestrogen, in establishing a need for oestrogen in early pregnancy in the bonnet monkey (*Macaca radiata*). *Journal of Reproduction and Fertility* **81**, 327-336.

Reddi, K., Illingworth, P.J., Saunders, P.T.K., Smith, K.B., Hillier, S.G. & Baird, D.T. (1990b). Expression of inhibin alpha subunit mRNA in the human corpus luteum is maintained throughout the luteal phase. *Journal of Endocrinology* **124**(Suppl), Abstract No.75.

Reddi, K., Wickings, E.J., Hillier, S.G. & Baird, D.T. (1989). Bioactive FSH and inhibin concentrations during ovulation induction in patients with polycystic ovarian disease. *Journal of Endocrinology* **123** (Suppl.), Abstract 26.

Reddi, K., Wickings, E.J., McNeilly, A.S., Baird, D.T. & Hillier, S.G. (1990a). Circulating bioactive follicle stimulating hormone and immunoreactive inhibin during the normal human menstrual cycle. *Clinical Endocrinology* **33**, 547-557.

Rehbein, M., Hillers, M., Mohr, E., Ivell, R., Morley, S., Schmale, H. & Richter, D. (1986). The neurohypophyseal hormones vasopressin and oxytocin. Precursor structure, synthesis and regulation. *Biological Chemistry Hoppe Seyler* **367**, 695-704.

Richards, J.S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S., Durica, J.M. & Goldring, N.B. (1987). Ovarian follicular development: from physiology to molecular biology. *Recent Progress in Hormone Research* **43**, 231-270.

Richardson, M.C. (1986). Hormonal control of ovarian luteal cells. *Oxford Reviews in Reproductive Biology* **8**, 321-378.

Richardson, M.C. & Masson, G.M. (1981). Stimulation by human chorionic gonadotrophin of oestradiol production by dispersed cells from human corpus luteum: comparison with progesterone production; utilization of exogenous testosterone. *Journal of Endocrinology* **91**, 197-203.

Rivier, C., Rivier, J. & Vale, W. (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science* **234**, 205-208.

Rivier, C., Roberts, V. & Vale, W. (1989). Possible role of luteinizing hormone and follicle stimulating hormone on modulating inhibin secretion and expression during the estrous cycle of the rat. *Endocrinology* **125**, 876-882.

Rivier, J., Spiers, J., McClintock, R., Vaughan, J. & Vale, W. (1985). Purification and partial characterization of inhibin from porcine follicular fluid. *Biochemical and Biophysical Research Communications* **133**, 120-127.

Roberts, V.J., Meunier, H., Plotsky, P.M., Rivier, C., Sawchenko, P.E. & Vale, W. (1989). Inhibin/activin proteins in pituitary and brain. In *'Neuroendocrine Regulation of Reproduction,'* pp269-277. Proceedings of Serono Symposium, Napa Valley, U.S.A. .

Robertson, D.M., (1990). The measurement of inhibin. *Reproduction, Fertility and Development* **2**, 101-105.

Robertson, D.M., de Vos, F.L., Foulds, L.M., McLachlan, R.I., Burger, H.G., Morgan, F.J., Hearn, M.T.W. & de Kretser, D.M. (1986). Isolation of 31kDa form of inhibin from bovine follicular fluid. *Molecular and Cellular Endocrinology* **44**, 271-277.

Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H. and de Kretser, D.M. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications* **126**, 220-226.

Robertson, D.M., Giacometti, M., Foulds, L.M., Lahnstein, J., Goss, N.H., Hearn, M.T.W, & de Kretser, D.M. (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. *Endocrinology* **125**, 2141-2149.

Robertson, D.M., Hayward, S., Irby, D., Jacobsen, J., Clarke, L., McLachlan, R.I. & de Kretser, D.M. (1988b). Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. *Molecular and Cellular Endocrinology* **58**, 1-8.

Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E., Hearn, M.T., Burger, H.G. & de Kretser, D.M. (1987). The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochemical and Biophysical Research Communications* **149**, 744-749.

Robertson, D.M., Tsonis, C.G., McLachlan, R.I., Handelsman, D.J., Leask, R., Baird, D.T., McNeilly, A.S., Hayward, S., Healy, D.L., Findlay, J.K., Burger, H.G. & de Kretser, D.M. (1988a). Comparison of inhibin immunological and *in vitro* biological activities in human serum. *Journal of Clinical Endocrinology and Metabolism* 67, 438-443.

Rodgers, R.J. & O'Shea, J.D. (1982). Purification, morphology and progesterone production and content of three cell types isolated from the corpus luteum of sheep. *Australian Journal of Biological Sciences* **35**, 441-455.

Rodgers, R.J., O'Shea, J.D. & Bruce, N.W. (1984). Morphometric analysis of the cellular composition of the ovine corpus luteum. *Journal of Anatomy* **138**, 757-769.

Rodgers, R.J., Stuchbery, S.J. & Findlay, J.K. (1989). Inhibin mRNAs in ovine and bovine ovarian follicles and corpora lutea throughout the oestrous cycle and gestation. *Molecular and Cellular Endocrinology* **62**, 95-101.

Ronnekleiv, O.K., Adelman, J.P., Weber, E., Herbert, E. & Kelly, M.J. (1987). Immunohistochemical demonstration of proGnRH and GnRH in the preoptic-basal hypothalamus of the primate. *Neuroendocrinology* **45**, 518-521.

Rothchild, I. (1965). Interrelations between progesterone and the ovary, pituitary and central nervous system in the control of ovulation and the regulation of progesterone secretion. *Vitamins and Hormones* **23**, 209-327.

Rothchild, I. (1981). The regulation of the mammalian corpus luteum. *Recent Progress in Hormone Research* **37**, 183-298.

Sander, H.J., van Leeuwen, E.C.M. & de Jong, F.H. (1984). Inhibin-like activity in media from cultured rat granulosa cells collected throughout the oestrous cycle. *Journal of Endocrinology* **103**, 77-84.

Sandow, J. (1983). The regulation of LHRH action at the pituitary and gonadal receptor level: a review. *Psychoneuroendocrinology* **8**, 277-297.

Sargent, E.L., Baughman, W.L., Novy, M.J. & Stouffer, R.L. (1988). Intraluteal infusion of a prostaglandin synthesis inhibitor, sodium meclofenamate, causes premature luteolysis in rhesus monkeys. *Endocrinology* **123**, 2261-2269

Sarker, D.K. & Fink, G. (1980). Luteinizing hormone releasing factor in pituitary stalk plasma from long-term ovariectomized rats: effect of steroids. *Journal of Endocrinology* **86**, 511-524.

Sasano, H., Okamoto, M., Mason, J.I., Simpson, E.R., Mendelson, C.R., Sasano, N. & Silverberg, S.G. (1989). 17α-hydroxylase and side chain cleavage P450 in the human ovary. *Journal of Reproduction and Fertility* **85**, 163-169.

Savard, K., Marsh, J.M. & Rice, B.F. (1965). Gonadotrophins and ovarian steroidogenesis. *Recent Progress in Hormone Research* **21**, 285-365.

Savoy-Moore, R.T., Schwartz, N.B., Duncan, J.A. & Marshall, J.C. (1980). Pituitary gonadotrophin-releasing hormone receptors during the rat estrous cycle. *Science* **209**, 942-944.

Savoy-Moore, R.T., Schwartz, N.B., Duncan, J.A. & Marshall, J.C. (1981). Pituitary gonadotrophin-releasing hormone receptors on proestrus: effects of pentobarbital blockade of ovulation in the rat. *Endocrinology* **109**, 1360-1364.

Sawchenko, P.E., Plotsky, P.M., Pfeiffer, S.W., Cunningham, E.T.Jr., Vaughan, J., Rivier, J & Vale, W. (1988). Inhibin β in central neural pathways involved in the control of oxytocin secretion. *Nature* **334**, 615-617.

Sawyer, C.H. (1978). History of the neurovascular concept of hypothalamohypophyseal control. *Biology of Reproduction* **18**, 325-328.

Scaramuzzi, R.J., Baird, D.T., Wheeler, A.G. & Land, R.B. (1973). The oestrus cycle of the ewe following active immunization against prostaglandin $F_{2\alpha}$. *Acta Endocrinologica [Suppl]* **177**, 318.

Scaramuzzi, R.J., Corker, C.S., Young, G. & Baird, D.T. (1974). Production of antisera to steroid hormones in sheep. In: *Steroid Immunoassay*, pp111-122. Eds. E.H.D. Cameron, S.G. Hillier, K. Griffiths.

Schaeffer, J.M., Liu, J., Hseuh, A.J. & Yen, S.S.C. (1984). Presence of oxytocin and arginine vasopressin in human ovary, oviduct and follicular fluid. *Journal of Clinical Endocrinology and Metabolism* **59**, 970-973.

Schams, D., Prokopp, S. & Barth, D. (1983). The effect of active and passive immunization against oxytocin on ovarian cyclicity in ewes. *Acta Endocrinologica* **103**, 337-344.

Schanbacher, B. (1988). Radioimmunoassay of inhibin. Serum responses to unilateral and bilateral orchidectomy. *Endocrinology* **123**, 2323-2330.

Schenken, R.S. & Hodgen, G.D. (1983) Follicle stimulating hormone induced ovarian hyperstimulation in monkeys: blockade of the luteinizing hormone surge. *Journal of Clinical Endocrinology and Metabolism* **57**, 50-55.

Schillo, K.K., Leshin, L.S., Kuehl, D. & Jackson, G.L. (1985). Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone during estradiol-induced luteinizing hormone surges in the ovariectomized ewe. *Biology of Reproduction* **33**, 644-652.

Schneyer, A.L., Mason, A.J., Burton, L.E., Ziegner, J.R.& Crowley, W.F. (1990). Immunoreactive inhibin α-subunit in human serum: implications for radioimmunoassay. *Journal of Clinical Endocrinology and Metabolism* **70**, 1208-1212.

Schoonmaker, J.N., Bergman, K.S., Steiner, R.A. & Karsch, F.J. (1984). Oestradiol-induced luteal regression in the rhesus monkey: evidence for an extra-ovarian site of action. *Endocrinology* **110**, 1708-1715.

Schwall, R.H., Mason, A.J., Wilcox, J.N., Bassett, S.G. & Zeleznik, A.J. (1990). Localization of inhibin/activin subunit mRNA's within the primate ovary. *Molecular Endocrinology* **4**, 75-79.

Schwall, R.H.& Niswender, G.D. (1985). Two types of steroidogenic luteal cells in the ewe: morphological and biochemical characteristics. In: *Implantation of the Human Embryo*. p31-45. R.G. Edwards, J.M. Purdy, P.C. Steptoe. Academic Press, London.

Schwartz, N.B. & Channing, C.P. (1977). Evidence for ovarian inhibin: suppression of the secondary rise in serum follicle stimulating hormone levels in proestrous rats by injection of porcine follicular fluid. *Proceedings of the National Academy of Science U.S.A.* **74**, 5721.

Scott, R.S. & Burger, H.G. (1981). Mechanism of action of inhibin. *Biology of Reproduction* **24**, 541-550.

Scott, R.S., Burger, H.G. & Quigg, H. (1980). A simple and rapid *in-vitro* bioassay for inhibin. *Endocrinology* **107**, 1536-1542.

Seeburg, P.H. & Adelman, J.P. (1984). Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* **311**, 666-668.

Seeburg, P.H., Mason, A.J., Stewart, T.A. & Nikolics, K. (1987). The mammalian GnRH gene and its pivotal role in reproduction. *Recent Progress in Hormone Research* **42**, 69-98.

Shackleton, C. H. L. (1974). Progesterone and oestradiol metabolism in the pregnant marmoset *(Callithrix jacchus). Journal of Steroid Biochemistry* **5**, 597-600

Shackleton, C. H. L. & Mitchell, F. L. (1975) The comparison of perinatal steroid endocrinology in simians with a view to finding a suitable animal model to study human problems. *Laboratory Animal Handbooks* **6,** 159-181

Sharpe, R.M., Swanston, I.A., Cooper, C.G., Tsonis, C.G. & McNeilly, A.S. (1988). Factors influencing the secretion of immunoreactive inhibin into testicular interstitial fluid in rats. *Journal of Endocrinology* **119**, 315-336.

Sheldrick, E.L., Mitchell, M.D. & Flint, A.P.F. (1980). Delayed luteal regression in ewes immunized against oxytocin. *Journal of Reproduction and Fertility* **59**, 37-42.

Sherwood, N.M., Chiappa, S.A. & Fink, G. (1976). Immunoreactive luteinizing releasing factor in pituitary stalk blood from female rats: sex steroid modulation of response to electrical stimulation of preoptic area or median eminence. *Journal of Endocrinology* **70**, 501-511,

Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S.Y., Ling, N. & Guillemin, R. (1988). Primary structure of the human follistatin precursor and its genomic organisation. *Proceedings of the National Academy of Sciences USA* **85**, 4218-4222.

Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A. & Ling, N. (1989). Follistatin gene expression in the ovary and extragonadal tissues. *Molecular Endocrinology* **3**, 651-659,

Shutt, D.A., Clark, A.H., Fraser, I.S., Goh, P., McMahon, G.P., Saunders, D.M. & Shearman, D.M. (1976). Changes in concentrations of prostaglandin F and steroids in human corpora lutea in relation to growth of the corpus luteum and luteolysis. *Journal of Endocrinology* **71**, 453-454.

Silverman, A.J., Antunes, J.L., Abrams, G.M., Nilaver, G., Thau, R., Robinson, J.A., Ferin, M. & Krey, L.C. (1982). The luteinizing hormone-releasing hormone pathways in rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemestrina*) monkeys. New observations on thick, unbedded sections. *Journal of Comparative Neurology* **211**, 309-317.

Snabels, M.C. & Kelch, R.P. (1979). Acute inhibitory effects of antiserum to gonadotrophin-releasing hormone in ovariectomized rats. *Neuroendocrinology* **29**, 34-41.

Song, T., Nikolics, K., Seeburg, P.H. & Goldsmith, P.C. (1986). GnRH prohormone immunoreactivity in the primate forebrain and hypothalamus (Abstract). *1st International Congress of Neuroendocrinology*, p89.

Soper, B.D. & Weick, R.F. (1980). Hypothalamic and extrahypothalamic mediation of pulsatile discharges of luteinizing hormone in the ovariectomized rat. *Endocrinology* **106**, 348-355.

Sotrel, G., Helvacioglu, A., Dowers, S., Scommegna, A. & Auletta, F.J. (1981). Mechanisms of luteolysis: Effect of oestradiol and $PGF_{2\alpha}$ on corpus luteum. *Americal Journal of Obstetrics and Gynaecology* **139**, 134-140.

Soules, M.R., McLachlan, R.I., Ek, M., Dahl, K.D., Cohen, N.L. & Bremner, W.J. (1989). Luteal phase deficiency: Characterization of reproductive hormones over the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **69**, 804- 812.

Soules, M.R., Steiner, R.A., Clifton, D.K., Cohen, N.L., Aksel, S. & Bremner, W.J. (1984). Progesterone modulation of pulsatile luteinizing hormone secretion in normal women. *Journal of Clinical Endocrinology and Metabolism* **58**, 378-383.

Spies, H.G. & Norman, R.L. (1975). Interaction of estradiol and LHRH on LH release in rhesus monkeys: Evidence for a neural site of action. *Endocrinology* **97**, 685-691.

Steinberger, A. & Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99**, 918-921.

Steinberger, E., Steinberger, A. & Vilar, O. (1966). Cytochemical study of Δ^5 3-β-hydroxysteroid dehydrogenase in testicular cells grown *in vitro*. *Endocrinology* **79**, 406-410.

Sternberger, L.A. (1979). *Immunocytochemistry*. Second Edition. John Wiley & Sons. New York.

Stewart, A.G., Milborrow, H.M., Ring, J.M., Crowther, C.E. & Forage, R.G. (1986). Human inhibin genes: genomic characterisation and sequencing. *FEBS Letters* **206**, 329-334.

Stillman, R.J., Williams, R.F., Lynch, A. & Hodgen, G.D. (1983) Selective inhibition of follicle stimulating hormone by porcine follicular fluid extracts in the monkey: effects on midcycle surges and pulsatile secretion. *Fertility and Sterility* **40**, 823-828.

Stouffer, R.L., Nixon, W.E. & Hodgen, G.D. (1979). Disparate effects of prostaglandins on basal and gonadotrophin-stimulated progesterone production by luteal cells isolated from rhesus monkeys during the menstrual cycle and pregnancy. *Biology of Reproduction* **20**, 897-903.

Stouffer, R.L., Nixon, W.E. & Hodgen, G.D. (1977). Oestrogen inhibition of basal and gonadotrophin-stimulated progesterone production by rhesus monkey luteal cells *in vitro*. *Endocrinology* **101**, 1157-1163.

Swanston, I.A., McNatty, K.P. & Baird, D.T. (1977). Concentration of prostaglandin F and steroids in the human corpus luteum. *Journal of Endocrinology* **73**, 155-

Sugino, H., Nakamura, T., Hasegawa, Y., Miyamoto, K., Abe, Y., Igarashi, M., Eto, Y., Shibai, H. & Titani, K. (1988). Erythroid differentiation factor can modulate follicular granulosa cell functions. *Biochemical and Biophysical Research Communications* **153**, 281-288.

Sugino, K., Nakamura, T., Takio, N., Titami, K., Miyamoto, K., Hasegawa, Y., Igarashi, M. & Sugino, H. (1989). Inhibin α-subunit monomer is present in follicular fluid. *Biochemical and Biophysical Research Communications* **159**, 1323-1329.

Tanabe, K., Gagliano, P., Channing, C.P., Nakamura, Y., Yoshimura, Y., Izuka, R., Fortury, A., Sulewski, J. & Rezao, N. (1983). Levels of inhibin-F activity and steroids in human follicular fluid from normal women and women with polycystic ovarian disease. *Journal of Clinical Endocrinology and Metabolism* **57**, 24-31.

Taya, K., Komura, H., Watanabe, G. & Sasamoto, S. (1989). Peripheral blood levels of immunoreactive inhibin during pseudopregnancy, pregnancy and lactation in the rat. *Journal of Endocrinology* **121**, 545-552.

Tierney, M.L., Goss, N.H., Tomkins, S.M., Kerr, D.B., Pitt, D.E., Forage, R.G., Robertson, D.M., Hearn, M.T.W & de Kretser, D.M. (1990). Physicochemical and biological characterization of recombinant human inhibin A. *Endocrinology* **126** 3268-3270.

Torney, A.H., Hodgson, Y.M., Forage, R. & de Kretser, D.M. (1989). Cellular localization of inhibin mRNA in the bovine ovary by in situ hybridization. *Journal of Reproduction and Fertility* **86**, 391-399.

Tsai, C.C. & Yen, S.S.C. (1971). Acute effects of intravenous infusion of 17β-estradiol on gonadotropin release in pre- and post-menopausal women. *Journal of Clinical Endocrinology and Metabolism* **32**, 766-761.

Tsonis, C.G., Hillier, S.G. & Baird, D.T. (1987b). Production of inhibin bioactivity by human granulosa-lutein cells: stimulation by LH and testosterone *in vitro*. *Journal of Endocrinology* **112**, R11-R14.

Tsonis, C.G., McNeilly, A.S. & Baird, D.T. (1986). Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine pituitary FSH secretion *in vitro*. *Journal of Endocrinology* **110**, 341-352.

Tsonis, C.G., McNeilly, A.S. & Baird, D.T. (1987a). Production and secretion of ovarian inhibin. In: *Inhibin-non-steroidal regulation of follicle stimulating hormone secretion.* pp203-218. Eds. H.G.Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi. New York: Raven Press.

Tsonis, C.G., Messinis, I.E., Templeton, A.A., McNeilly, A.S. & Baird, D.T. (1988). Gonadotrophic stimulation of inhibin secretion into peripheral blood by the human ovary during the follicular and early luteal phase of the cycle. *Journal of Clinical Endocrinology and Metabolism* **66** (5), 915-921.

Tsonis, C.G., Pearson, M., Hungerford, J., Borchers, C.E., Greenwood, P.E., Forage, R.G., Doughton, B., Findlay, J.K. (1989). Immunizing sheep with recombinant inhibin α-subunit increases the number of lambs born. *Proceedings of the Australian Society for Reproductive Biology* **21**, Abstract no.99.

Tsonis, C.G., Quigg, H., Lee, V.W.K., Leversha, L., Trounson, A.O. & Findlay, J.K. (1983). Inhibin in individual ovine follicles in relation to diameter and atresia. *Journal of Reproduction and Fertility* **67**, 83-90.

Ueno, N., Ling, N., Ying, S.Y., Esch, F., Shimasaki, S. & Guillemin, R. (1987). Isolation and partial characterization of follistatin: a single chain Mr 35000 monomeric protein that inhibits the release of follicle stimulating hormone. *Proceedings of the National Academy of Sciences USA* **84**, 8282-8286.

Ursely, J., Darbon, J.M. & Lemarie, P. (1981). Aromatization of testosterone in large and small bovine luteal cells. Conflicting results between radioimmunoassay and cocrystallization data. *Steroids* **38**, 271-279.

Ursely, J. & Leymarie, P. (1979). Varying responses of two luteal cell types isolated from bovine corpus luteum. *Journal of Endocrinology* **83**, 303.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. & Spiess, J. (1986). Purification and characterization of an FSH releasing protein from porcine follicular fluid. *Nature* **326**, 776-779.

Van Orden, D.E., Swanston, J.A., Clancey, C.J. & Farley, D.H. (1977). Plasma prostaglandins in the normal menstrual cycle. *Obstetrics and Gynaecology* **50**, 639-643.

Van Vugt, D.A., Bakst, G., Dyrenfurth, I. & Ferin, M. (1983). Naloxone stimulation of LH secretion in the female monkey: influence of endocrine and experimental conditions. *Endocrinology* **113**, 1858-1864.

Veldhuis, J.D. & Demers, L.M. (1986). An inhibitory role for protein kinase C pathway in ovarian steroidogenesis. *Biochemical Journal* **239**, 505-511.

Vickery, B.H. (1986). Pharmacology of LHRH antagonists. In: *Pharmacology and clinical use of inhibitors of hormone secretion and action.* p385. Eds. B.J.A. Furr, A. Wakeling. Bailliere Tindall, Eastbourne.

Vickery, B.H. & Nestor, J.J.Jr. (1987). Luteinizing hormone-releasing hormone analogs: development and mechanism of action. *Seminars in Reproductive Biology* **5**, 353-369.

Vijayakumar, R. & Walters, W.A.W. (1983). Human luteal tissue prostaglandins, 17β-oestradiol and progesterone in relation to the growth and senescence of the corpus luteum. *Fertility and Sterility* **39**, 298-303.

Walker, K.J., Turkes, A.O., Turkes, A., Zwink, R., Peacock, C., Buck, A.C., Peeling, W.B. & Griffiths, K. (1984). Treatment of patients with advanced cancer of the prostate using a slow-release (depot) formulation of the LHRH agonist ICI 118630 (Zoladex). *Journal of Endocrinology* **103**, R1-R4.

Walker, K.J., Turkes, A., Williams, M.R., Blamey, R.W. & Nicholson, R.I. (1986). Preliminary endocrinological evaluation of a sustained-release formulation of the LH-releasing hormone agonist D-Ser(Bu^t)^6 Azgly^{10}LHRH in premenopausal women with advanced breast cancer. *Journal of Endocrinology* **111**, 349-353.

Wardlaw, S.L., Wehrenberg, W.B., Ferin, M., Antunes, J.L. & Frantz, A.G. (1982). Effect of sex steroids on β-endorphin in hypophyseal portal blood. *Journal of Clinical Endocrinology and Metabolism* **55**, 877-881.

Wathes, D.C., Pickering, B.T., Swann, R.W., Porter, D.G., Hull, M.G.R. & Drife, J.O. (1982). Neurohypophyseal hormones in the human ovary. *Lancet* **ii**, 410-412.

Wathes, D.C. & Swann, R.W. (1982). Is oxytocin an ovarian hormone? *Nature* **297**, 225-227.

Watkins, W.B. (1983). Immunohistochemical localization of neurophysin and oxytocin in the sheep corpus luteum. *Neuropeptides* **4**, 51-54.

Webley, G.E., Knight, P.G., Given, A. & Hodges, J.K. (1991). Increased concentrations of immunoreactive inhibin during conception in the marmoset monkey: suppression with an LHRH antagonist and cloprostenol. *Journal of Endocrinology* in press.

Weick, R.F., Pitelka, V. & Thompson, D.L. (1982). Separate negative feedback effects of estrogen on the pituitary and the central nervous system in the ovariectomized rhesus monkey. *Endocrinology* **112**, 1862-1864.

Weinberger, G.F., Surmann, F.J., Akhtar, F.B., Shah, G.V., Vickery, B.H. & Nieschlag, E. (1984). Reversible inhibition of testicular function by a gonadotrophin hormone releasing hormone antagonist in monkeys (*Macaca fascicularis*). *Fertility and Sterility* **42**, 906-914.

Wentz, A.C. & Jones, G.S. (1973). Transient luteolytic effect of prostaglandin $F_{2\alpha}$ in the human. *Obstetrics and Gynaecology* **42**, 172-181.

Wehrenberg, W.W., Wardlaw, S.L., Frantz, A.G. & Ferin, M. (1982). β-endorphin in hypophyseal portal blood: variations throughout the menstrual cycle. *Endocrinology* **111**, 879-881.

White, R.F., Hertig, A.T., Rock, J. & Adams, E. (1951). Histological and histochemical observations on the corpus luteum of human pregnancy with special reference to corpora lutea associated with early normal and abnormal ova. *Contributions to Embryology* **34**, 55-74.

Wildt, L., Hausler, A., Marshall, J., Hutchison, J.S., Plant, T.M., Belchetz, P.E. & Knobil, E. (1981). Frequency and amplitude of gonadotrophin-releasing hormone stimulation and gonadotrophin secretion in the rhesus monkey. *Endocrinology* **109**, 376-385.

Wiley, C.A. & Esterly, J.R. (1976). Observations on the human corpus luteum: histochemical changes during development and involution. *American Journal of Obstetrics and Gynaecology* **125**, 514-519.

Wilks, J.W., Forbes, K.K. & Norland, J.F. (1972). Synthesis of prostaglandin $F_{2\alpha}$ by the ovary and uterus. In: *Prostaglandins*. p42-55 Ed. E.M. Souterine Mt Kitso, NY: Futura Publishing Co.

Williams, M.T., Roth, M.S., Marsh, J.M. & Lemaire, W.J. (1979). Inhibition of human chorionic gonadotrophin-induced progesterone synthesis by oestradiol in isolated human luteal cells. *Journal of Clinical Endocrinology and Metabolism* **48**, 437-440.

Woodruff, T.K., Meunier, H., Jones, P.B.C., Hsueh, A.J.W. & Mayo, K.E. (1987). Rat inhibin: molecular cloning of α- and β- subunit complementary deoxyribonucleic acids and expression in the ovary. *Molecular Endocrinology* **1**, 561-568.

Xiao, S., Findlay, J.K. & Robertson, D.M. (1990). The effect of bovine activin and FSH suppressing protein/follistatin on FSH-induced differentiation of rat granulosa cells in vitro. *Molecular and Cellular Endocrinology* **69** (1), 1-8.

Yamaji, T., Dierschke, D.J., Bhattacharya, A.N. & Knobil, E. (1972). The negative feedback control by oestradiol and progesterone of LH secretion in the ovariectomized rhesus monkeys. *Endocrinology* **90**, 771-777.

Yen, S.S.C., Llerena, O., Little, B.& Pearson, O.H. (1968). Disappearance rates of endogenous luteinizing hormone and chorionic gonadotrophin in man. *Journal of Clinical Endocrinology and Metabolism* **28**, 1763-1767.

Yen, S.S.C. & Tsai, C.C. (1971). The biphasic pattern in the feedback action of ethinyl estradiol on the release of pituitary FSH and LH. *Journal of Clinical Endocrinology and Metabolism* **33**, 882-887.

Yohkaichiya, T., Fukaya, T., Hoshiai, H., Yajima, A. & de Kretser, D.M. (1989). Inhibin: a new circulating marker of hydatidiform mole ? *British Medical Journal* **298**, 1684-1686.

Yu, J., Shao, Li-en, Lemas, V., Yu, A.L., Vaughan, J., Rivier, J. & Vale, W. (1987). Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* **330**, 765-767.

Zeleznik, A.J., Hutchison, J.S. & Schuler, H.M. (1987). Passive immunization with anti-oestradiol antibodies during the luteal phase of the menstrual cycle potentiates the perimenstrual rise in serum gonadotrophin concentrations and stimulates follicular growth in the cynomologus monkey (*Macaca fascicularis*). *Journal of Reproduction and Fertility* **80**, 403-410.

Zhiwen, Z., Carson, R.S., Herington, A.C., Lee, W.K. & Burger, H.G. (1987a). Follicle-stimulating hormone and somatomedin C stimulate inhibin production by rat granulosa cells *in vitro*. *Endocrinology* **120**, 1633-1638.

Zhiwen, Z., Herington, A.C., Carson, R.S., Findlay, J.K. & Burger, H.G. (1987b). Direct inhibition of rat granulosa cell inhibin production by epidermal growth factor *in vitro*. *Molecular and Cellular Endocrinology* **54**, 213-220.

Zhiwen, Z., Carson, R.S. & Burger, H.G. (1988a). Selective control of rat granulosa cell inhibin production by FSH and LH *in vitro*. *Molecular and Cellular Endocrinology* **58**, 161-166.

Zhiwen, Z., Findlay, J.K., Carson, R.S., Herington, A.C. & Burger, H.F. (1988b). Transforming growth factor-β enhances basal and FSH stimulated inhibin production by rat granulosa cells *in vitro*. *Molecular and Cellular Endocrinology* **58**, 161-166.