The Role of Inhibin and Oestradiol in the Control of Gonadotrophin Secretion in the Ewe

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Doctor of Philosophy

University of Edinburgh

1990

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Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

G. E. Mann
October 1990

Acknowledgements

I would like to thank the Faculty of Medicine, University of Edinburgh, for granting me the postgraduate research fellowship which allowed these studies to be undertaken. These studies were performed under the supervision of Prof. David Baird and Dr. Alan McNeilly, to whom I would like to express my gratitude for their assistance and encouragement. I would also like to express my gratitude to Dr. Bruce Campbell for assistance with various aspects of my work. Thanks are also due to Miss Norah Anderson and the staff of the Marshall Building for their technical assistance, and to a number of people who have assisted during the performance of experiments, in particular Miss Wendy Crow and Dr Anna Glasier. I would also like to thank Miss Rosemary Leask for assistance with the inhibin bioassay and Mr Tom McFetters and Mr Ted Pinner for advice on graphics. Finally I would like to thank anybody in the Centre for Reproductive Biology who has helped in any way with these studies.

Abstract

Inhibin is an ovarian glycoprotein which by definition causes the suppression of gonadotrophin production, preferentially that of FSH. In the sheep inhibin is secreted by the ovary, but there is confusion as to the actual ovarian source(s). As well as uncertainty over the source of inhibin, the physiological role of inhibin in the control of gonadotrophin secretion is unclear. It is well established that inhibin can suppress pituitary FSH production, but the physiological importance of this action requires further investigation. The main aims of the studies described in this thesis were firstly to determine the source of inhibin from the ovary of the sheep, and secondly to investigate the physiological role of inhibin in the control of gonadotrophin production, particularly that of FSH.

The source of ovarian inhibin production was investigated by measuring inhibin secretion directly from the ovary *in vivo*, and by individual follicles *in vitro*. Inhibin secretion did not differ between animals at different stages of the luteal and follicular phase of the oestrous cycle. The secretion rate of inhibin was unaffected by the presence or absence of luteal tissue suggestion that, in the sheep, the corpus luteum does not produce significant quantities of inhibin. The results of these studies indicated that, like oestradiol, the majority of inhibin is produced by large (≥ 3mm) antral follicles. However, while most oestradiol was secreted by the large oestrogenic follicle(s), a significant amount of inhibin was also produced by large non - oestrogenic atretic follicles and by small antral follicles.

A series of experiments involving passive immunisation against inhibin and/or oestradiol were then undertaken to investigate the relative importance of these two hormones in the control of gonadotrophin production. Peripheral LH concentrations were unaffected by immunisation against inhibin, and in further experiments administration of inhibin in "steroid stripped" ovine follicular fluid was shown to have no effect on the timing or magnitude of the oestradiol benzoate-induced LH surge in ovariectomised ewes, or on the concentration of LH following acute ovariectomy. In the passive immunisation studies, injection of antibodies to inhibin or oestradiol resulted in a highly significant, though transitory rise in the peripheral plasma concentration of FSH during both the luteal and follicular phases of the oestrous cycle, while combined immunisation against both hormones resulted in a significantly larger rise in FSH concentration of similar size to that seen following acute ovariectomy. Furthermore, treatment with physiological quantities of inhibin or oestradiol was found to partly prevent the rise in FSH concentration seen following acute ovariectomy, while a combined treatment with both hormones completely prevented this rise. Finally, immunisation against inhibin or oestradiol was shown to cause a large increase in the

number of follicles per ovary, resulting in an increase in ovarian inhibin secretion following immunisation against oestradiol, and an increase in ovarian oestradiol secretion following immunisation against inhibin. These results indicate that inhibin plays an important physiological role in the control of FSH secretion during both the luteal and follicular stages of the sheep oestrous cycle, and suggest that inhibin and oestradiol act together in the control of FSH secretion.

Chapter 1

Literature Review

1.1. The Ovine Oestrous Cycle

The oestrous cycle of the ewe lasts approximately 17 days, with oestrus occurring on day 0 and ovulation on day 1. The cycle can be divided into a luteal phase, lasting from day 2 - 13, and a follicular phase or periovulatory period lasting from day 14 (3 days before oestrous) to ovulation on day 1.

1.1.1. The Luteal Phase

Following ovulation a corpus luteum forms which secretes increasing amounts of progesterone as the luteal phase progresses, culminating in a plateau of progesterone concentration between days 6 and 12 (Pant et al., 1977) (Fig.1.1). Immediately following ovulation no follicles > 2mm are present in the ovaries as a result of the widespread atresia induced in all non ovulatory follicles > 2mm by the LH surge. However, by day 2 or 3 one or two large antral follicles develop, as indicated by the rise in the secretion rate of oestradiol (Holst et al., 1972), and from this time on fluctuations in the secretion of oestradiol and androgens can be detected as large antral follicles develop and undergo atresia (Turnbull et al., 1977). These fluctuations in oestradiol secretion by developing follicles are accompanied by fluctuations in the peripheral concentration of FSH, which is suppressed during periods of peak oestradiol production. While the concentration of FSH shows no consistent trend during the luteal phase, the concentration of LH gradually declines, reaching low levels by day 13 (Fig. 1.1). The secretion of LH is pulsatile with pulses occurring at intervals of approximately 3 - 10h during the luteal phase, each of these pulses of LH stimulating the secretion of oestradiol and androgens by the ovary (Baird et al., 1976).

Pulses of LH are released from the pituitary in response to pulses of GnRH from the hypothalamus, each pulse of LH in the peripheral plasma being preceded by a pulse of GnRH in the blood of the hypothalamo - hypophyseal portal vessels (Clarke & Cummins, 1982). FSH is also secreted by the pituitary, but unlike the secretion of LH, FSH secretion does not appear to be pulsatile in the sheep. While the existence of a separate releasing factor for FSH has been postulated the secretion of FSH is probably under the control of GnRH. This idea is supported by the fact that neutralisation of GnRH action on the pituitary by active immunisation (McNeilly et al., 1986), GnRH agonist treatment (McNeilly & Fraser, 1987), or pituitary stalk disconnection

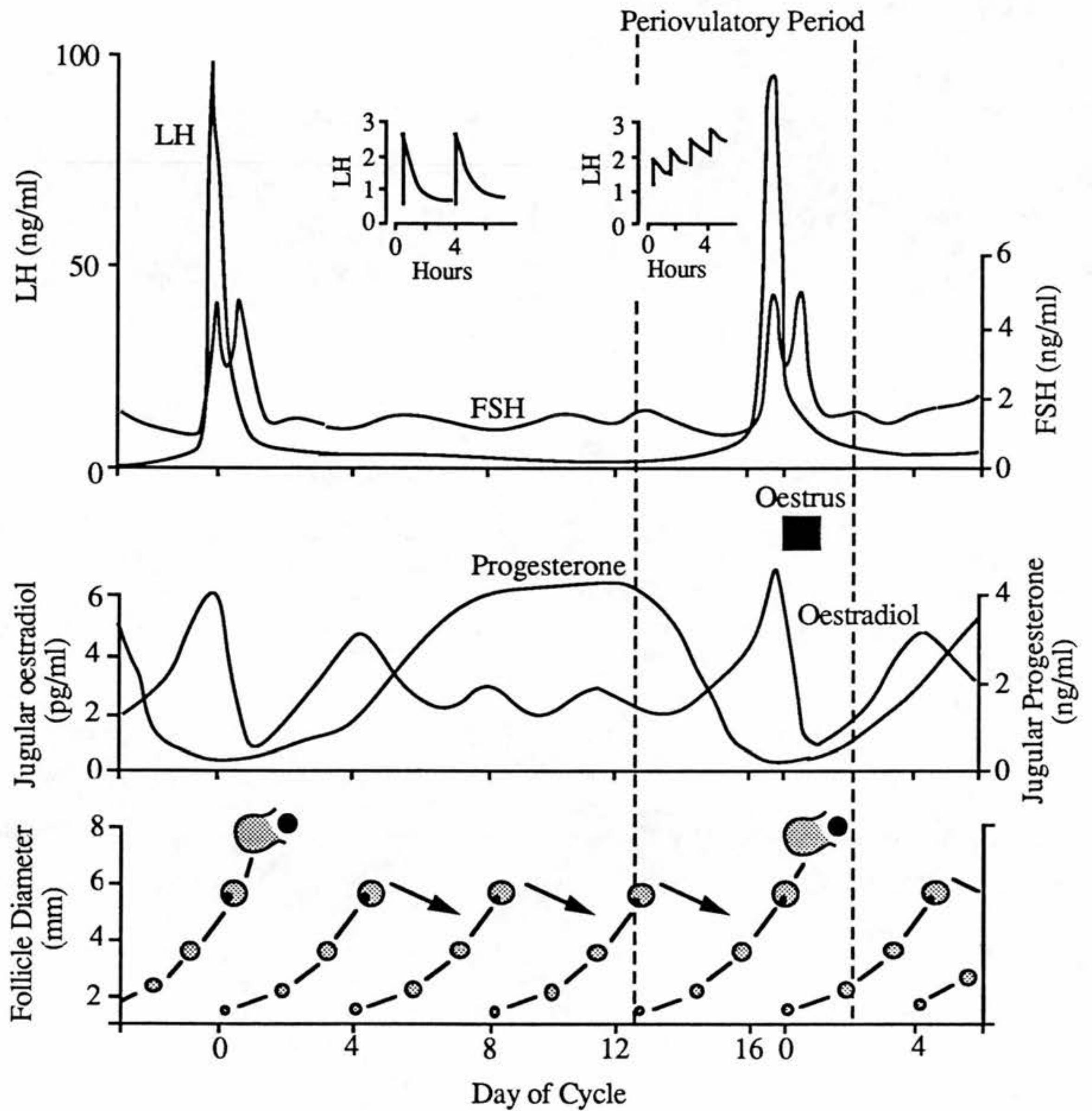

Fig. 1.1.
Hormonal and follicular changes during the oestrous cycle of the ewe centred around the onset of oestrus on day 0. Based on Baird & McNeilly (1981)

(Clarke,1987) results in a decline in FSH secretion. Furthermore, in hypothalamo - pituitary disconnected sheep administration of GnRH stimulates secretion of FSH, this secretion declining when treatment is stopped (Clarke et al., 1986). While active immunisation against GnRH results in a decline in FSH secretion, passive immunisation, while suppressing LH pulsatility, does not result in a decline in the secretion of FSH, which remains constant, or may even rise due to the fall in the secretion of oestradiol (McNeilly et al., 1984). Less than 10% of the pituitary content of LH is released during each 24h period compared with 60 - 80% of the FSH content (McNeilly, 1988), and so it appears that FSH secretion may be more closely related to the rate of synthesis than that of LH, which depends on synthesis and release.

During the luteal phase LH secretion is regulated by the negative feedback of progesterone and oestradiol, secreted by the ovary (Baird & Scaramuzzi, 1976a; Karsch et al., 1977). Oestradiol appears to suppress LH pulse amplitude (Goodman & Karsch, 1980), while progesterone acts at the hypothalamic level to suppress LH pulse frequency (Goodman & Karsch1980; Goodman et al., 1981a). The idea that oestradiol acts at the pituitary level to reduce pituitary responsiveness to GnRH is supported by the fact that oestradiol treatment has been shown to reduce the pituitary response to exogenous GnRH (Goodman & Karsch1980), and in hypothalamo - pituitary disconnected ewes GnRH secretion from the hypothalamus has been shown to continue after exogenous oestradiol treatment, while LH secretion from the pituitary ceases (Clarke & Cummins, 1982). This effect of progesterone appears to be exerted on the hypothalamus as treatment with progesterone does not prevent the stimulation of LH secetion by exogenous GnRH (Cumming et al., 1973a). During the luteal phase oestradiol appears to enhance the ability of progesterone to suppress LH release (Goodman et al., 1980), and so it seems that these two hormones act synergistically to control LH secretion. While LH release is controlled by the interaction of progesterone and oestradiol, FSH secretion is thought to be under the dual control of oestradiol and inhibin (Martin et al., 1988), with progesterone playing little if any role.

Due to the presence of high concentrations of progesterone during the luteal phase LH pulse frequency is maintained at a low level effectively reducing the stimulation of follicular oestradiol production. As a result of this, while large antral follicles are present throughout the luteal phase, ovulation does not occur as there is insufficient oestradiol secretion to generate an LH surge. Progesterone also appears to have a direct role in inhibiting the generation of an LH surge during the luteal phase as the hormone has been shown to inhibit the positive feedback effect of oestradiol in generating an LH surge in ovariectomised ewes (Scaramuzzi et al., 1971) and in anoestrous ewes (Howland et al., 1978). This fact would help account for the inability

of oestradiol to generate a preovulatory gonadotrophin surge until after luteal regression.

Progesterone is also important in priming the uterus for $PGF_{2\alpha}$ production (Baird, 1978a), the feedback loop between the ovary and the uterus regulating the length of the luteal phase. The low levels of LH on days 12 - 13 results in the corpus luteum becoming increasingly sensitive to the luteolytic effects of this uterine $PGF_{2\alpha}$ which in turn results in a decrease in progesterone production. This decline in progesterone production then leads to an increase in uterine $PGF_{2\alpha}$ release. The importance of $PGF_{2\alpha}$ in luteal regression is demonstrated by the fact that active immunisation against $PGF_{2\alpha}$ results in complete failure of luteal regression (Scaramuzzi & Baird, 1976), while injections of $PGF_{2\alpha}$ analogues have become widely used as a method of inducing premature luteal regression. Oxytocin is produced by the ovine corpus luteum (Wathes & Swann, 1982) and appears also to be involved in the process of luteal regression. The importance of oxytocin in this process is supported by the fact that both active (Sheldrick et al., 1980) and passive (Schams et al., 1983) immunisation against oxytocin results in delayed luteal regression. It is thought that oxytocin binds to newly developed receptors on the uterine endometrium stimulating $PGF_{2\alpha}$ production which in turn stimulates further oxytocin release. Continuous infusion of oxytocin has been shown to prevent the induction of these uterine receptors and hence delay luteal regression in the sheep (Flint & Sheldrick, 1985). Most ovarian oxytocin production occurs during the first half of the luteal phase and the hormone is therefore thought to have other roles in the sheep including the control of reproductive tract motility (Wathes, 1984). Oestradiol is probably also involved in the process of luteal regression, injections of oestradiol during the mid luteal phase resulting in premature luteal regression in the sheep (Hawk & Bolt, 1970). Administration of oestradiol has been shown to stimulate uterine $PGF_{2\alpha}$ production (Hixon & Flint, 1987), resulting in inhibition of progesterone production and luteal regression. While the exact mechanism underlying luteal regression remains uncertain, it seems that luteal oxytocin, as well as perhaps oestradiol, stimulate the production of uterine $PGF_{2\alpha}$, which appears to be the luteolytic factor in the sheep.

1.1.2. Luteal Regression to the LH Surge

As a result of the fall in progesterone concentration following luteal regression there is an increase in LH pulse frequency up to around one pulse every hour (Baird, 1978b) and though pulse amplitude decreases there is also an increase in basal concentration (Fig. 1.1). This increase in LH pulse frequency and/or basal

concentration appears to be important in stimulating the large rise that is seen at this time in the secretion of oestradiol by the the follicles destined to ovulate. Oestradiol may, itself, also be involved in enhancing LH pulse frequency at this time (Karsch et al., 1983). The rise in the secretion of oestradiol is accompanied by a smaller rise in the secretion of androgens and so the oestogen to androgen ratio rises, presumably as a result of increased aromatisation of androgen to oestradiol by the preovulatory follicle(s). The importance of the fall in progesterone concentration in causing the rise in LH and oestradiol secretion is supported by the observation that a sustained rise in LH release does not occur until after progesterone levels begin to decline, and by the fact that enucleation of the corpus luteum at various stages of the luteal phase, a process which dramatically reduces peripheral plasma progesterone levels, induces this rise in LH secretion. Furthermore, administration of progesterone at the time of luteal regression, via a progesterone - releasing implant, prevents the rise in both LH concentration and oestradiol secretion (Karsch et al., 1979; Baird & Scaramuzzi, 1976). Infusion of LH into the ovary via the ovarian artery results in a rapid increase in oestradiol and androgen release (McCracken et al., 1969) providing evidence for the role of LH in stimulating the follicular phase rise in oestradiol secretion. This idea is supported by the finding that infusion of LH into anoestrous ewes at a rate similar to the preovulatory rise in LH causes a rise in oestradiol secretion, and will elicit an LH surge (Karsch et al., 1979). The decrease in LH pulse amplitude seen at this time is probably the result of direct effects of the increased levels of oestradiol feedback on the pituitary (Baird & McNeilly, 1981).

The rise in oestradiol production stimulates uterine $PGF_{2\alpha}$ production, adding to the process of luteal regression, as well as causing a fall in the secretion of FSH which is thought to be important in the process of follicle selection (see Section 1.2.5). Inhibin, the peripheral concentration and secretion rate of which have been reported by some workers to increase at this time (Campbell et al., 1990a; Findlay et al., 1990), may also be involved in causing the suppression of FSH secretion seen at this time (see Section 1.4). The rise in oestradiol secretion is also important in inducing oestrous behaviour and in generating the preovulatory gonadotrophin surge. The importance of oestradiol in generating this LH surge is demonstrated by the fact that injection of oestradiol will generate a similar surge of LH secretion in seasonally anoestrous (Goding et al., 1969; Radford et al., 1971) or ovariectomised (Radford et al., 1971; Scaramuzzi et al., 1971) ewes, while active (Pant & Rawlings, 1973; Rawlings et al., 1978) or passive (Rawlings et al., 1979) immunisation against oestradiol during the follicular phase will prevent both oestrus and the LH surge. The mechanism by which the gonadotrophin surge is actually initiated is not known. However, as a single

injection, multiple injections, or a continuous infusion of oestradiol will induce a gonadotrophin surge (Martin, 1984), it seems unlikely that the pulsatile nature of oestradiol release is a key issue, and that the total amount of oestradiol present in the peripheral circulation is the important factor.

In summary, during the period following luteal regression the fall in the peripheral concentration of progesterone results in an increase in LH pulse frequency which stimulates the production of oestradiol. The increase in the concentration of oestradiol in the peripheral circulation then causes a decline in the secretion of FSH, perhaps in conjunction with inhibin, as well as generating the preovulatory gonadotrophin surge by positive feedback.

1.1.3 The Preovulatory Gonadotrophin Surge

Between 40 and 60h following the initiation of luteal regression a large rise in the peripheral concentrations of both LH and FSH occurs (Fig. 1.1). This rise in gonadotrophin release results from the rise in oestradiol secretion seen at this time, and it is likely that the positive feedback effects of oestradiol are exerted at the level of both the pituitary and the hypothalamus (Baird & McNeilly, 1981). In the ewe, as in many species, the pituitary is more responsive to GnRH stimulation in terms of FSH and LH release during the period prior to the preovulatory gonadotrophin surge than at any other stage of the cycle (Reeves et al., 1971, Hooley et al., 1974), this increase in sensitivity probably occurring in response to the rise in oestradiol. In ovariectomised ewes treated with oestradiol there is initially a period of LH suppression followed by a large increase in LH secretion (an LH surge) resulting from the positive feedback effects of oestradiol. Caraty et al. (1989) have shown that during the period of LH suppression there is a marked decrease in the frequency and amplitude of GnRH pulses, while during the period of positive feedback there is a large surge in the concentration of GnRH due primarily to a large increase in the frequency of GnRH pulses. An increase in GnRH pulse frequency during oestradiol - stimulated LH surges in ovariectomised ewes has also been shown by Clarke & Cummins (1985). While the responses seen were variable, increases in GnRH have also been shown at the time of the LH surge in oestradiol - treated anoestrous ewes and in normal cyclic ewes (Clarke, 1987). It appears, therefore, that the LH surge is due to both an increase in GnRH secretion from the hypothalamus and an increase in pituitary responsiveness to GnRH.

During the LH surge there is a rapid increase from basal concentrations to a peak within 4 - 8h, with concentrations returning to basal levels within 10h, resulting in a surge of approximately 10 - 18h duration (Land et al., 1973; Martin, 1984). The

surge usually occurs within 4 - 8h of the onset of oestrus (Acritopoulou et al., 1977; Land et al., 1973), with ovulation occurring approximately 21 - 26h later (Cumming et al., 1973b; Wheatley & Radford, 1969). During the initial stages of the LH surge there is a stimulation of steroid secretion by the dominant follicle (Baird & McNeilly, 1981). The secretion of androstenedione and testosterone is stimulated more than that of oestradiol and so the oestrogen to androgen ratio, which has increased over the course of the follicular phase, shows a marked decline. The LH surge then results in a marked decline in the secretion of first oestradiol and then androgens (Baird & McNeilly, 1981; Campbell et al., 1990a). As the secretion of oestradiol is affected before that of androgens loss of aromatase activity is probably involved in the early stages of this suppression. This idea is supported by the fact that the treatment of follicles *in vitro,* or ovaries *in vivo,* with quantities of LH equivalent to the LH surge results in a marked reduction in oestradiol secretion (Moor, 1974). The eventual suppression of all steroid production is probably the result of the loss of LH receptor activity, and following the LH surge a fall in LH receptors has been shown in both sheep (Webb & England, 1982) and cattle (Ireland & Roche, 1982).

1.1.4 LH Surge to Ovulation

The LH surge induces morphological changes in the preovulatory follicles which eventually culminate in ovulation and corpus luteum formation, while inducing atresia in all other follicles > 2mm. As a result of the loss of steroid production from the dominant follicle(s) and the atresia induced in other large antral follicles steroid secretion by the ovary at the time of ovulation is lower than at any other stage of the oestrous cycle. Despite this fact, LH secretion remains low as a result of pituitary depletion following the LH surge. There is, however, a second surge of FSH secretion of similar magnitude and duration to that seen coincidental with the LH surge at around the time of ovulation, some 18 - 24h after the first peak (Salamonson et al., 1973). The first FSH peak can be prevented by treatment with antisera to GnRH (Narayana & Dobson, 1979) suggesting that it results from an increase in the secretion of GnRH. This second peak cannot, however, be prevented using antisera to GnRH, suggesting that it occurs independently of an increase in GnRH secretion. Due to the low concentrations of steroids at this time it would seem likely that this second FSH peak is caused by a low level of steroid negative feedback at the pituitary level resulting in an increased sensitivity to GnRH. It has been suggested that inhibin may play a role in the control of FSH secretion following the LH surge, but at the time of the second FSH peak there is a marked rise in the secretion rate and peripheral concentration of

immunoactive inhibin (Campbell et al., 1990a). This finding would suggest that inhibin is not important in the suppression of FSH during this period. However, it is possible that the inhibin measured at this time could be a form of the molecule which is biologically inactive (see Section 1.4).

It has been suggested that this rise in FSH may be involved in controlling the number of potential ovulatory follicles that are stimulated to develop by the time of the next oestrus, as appears to be the case in rodents (Sheela Rani & Moudgal, 1977). However, in the sheep, induction of luteal regression at any time after day 4 of the cycle results in a similar ovulation rate (Baird & McNeilly, 1981) and so it seems unlikely that ovulation rate is predetermined as early in the cycle as this second FSH peak. In cattle, abolition of the second peak of FSH with inhibin from steroid extracted bovine follicular fluid has been shown to result in a delay in the appearance of large antral follicles in both the first and second waves of follicle growth, while not affecting cycle length (Turzillo & Fortune, 1990). It would appear, therefore, that this second FSH peak is important in reinitiating follicle development at the start of the oestrous cycle and in controlling the dynamics of follicle growth during the early stages of the cycle, but not in determining the eventual ovulation rate.

1.2. Follicle Development & Function

1.2.1. Follicle Growth

In the adult ewe the ovary contains between 12 000 and 18 000 small preantral follicles as well as 100 to 400 growing follicles (Cahill et al., 1979; McNatty et al., 1982) and it is thought that 3-4 follicles enter this growth phase each day (Turnbull et al., 1977), at a diameter of 0.03-0.06 mm (Cahill & Mauleon, 1981). Of the large number of follicles that commence growth only a very small proportion will actually go on to ovulate, the vast majority facing eventual atresia.

The mechanism of growth initiation is not known though the process appears to occur independantly of gonadotrophins as hypophysectomy, while causing a reduction in the number of antral follicles, does not affect the number of preantral follicles in ewes (Dufour et al., 1979). The rate of growth initiation has been found to differ between breeds of sheep, with more follicles entering the growth phase in more prolific breeds of sheep (Cahill et al., 1979), and with season, more follicles entering the growth phase during anoestrus (Cahill & Mauleon, 1980).

Follicle growth rate is slow up to a diameter of around 0.4 mm (Turnbull et al., 1977) and then accelerates. This acceleration in growth rate is due, in part, to the formation of an antrum, which occurs at a size of around 0.2 mm in the sheep (Mariana & Machado, 1976; Cahill et al., 1979), as once the antrum has formed further growth results from follicular fluid accumulation as well as granulosa and theca cell proliferation. The actual rate of division of granulosa cells (mitotic index) rises to a maximum at a diameter of 1-2.5 mm (Turnbull et al., 1977), and then decreases slowly to reach zero in the pre-ovulatory follicle (Cahill, 1981). The entire growth phase, from growth initiation at a diameter of 0.06 mm to the preovulatory stage (6-8 mm) is though to take about 6 months, and comprises 130 days of preantral growth (Cahill & Mauleon, 1980), followed by a period of around 34-45 days of further, more rapid growth following antrum formation (Turnbull et al., 1977; Cahill & Mauleon, 1980). During the latter stages of antral growth the follicle takes about 5 days to increase in diameter from 0.5 to 2.0 mm and a further 4 days to reach 4.5-5.0 mm, the maximum size seen in the absence of the pre-ovulatory gonadotropic stimulus (Turnbull et al., 1977). The slower growth rate before antrum formation is reflected in the presence of twice as many preantral as antral follicles in the sheep ovary (Cahill, 1981; Webb & Gauld, 1985). In the sheep waves of follicle growth and atresia are thought to occur throughout the cycle, follicles only avoiding the degenerative process of atresia if the development of FSH and LH receptors coincides with suitable levels of FSH and LH in the circulation. While different studies have demonstrated different numbers of waves and waves at different times during the oestrous cycle the evidence points to the existence of 3 waves of follicle growth during each cycle (Smeaton & Robinson, 1971; Cox et al., 1971; Mattner & Braden, 1972).

The actual growth rate of follicles does not seem to be affected by breed, stage of cycle or season (Cahill & Mauleon, 1980), though more prolific breeds appear to contain fewer very small follicles ($\leq$ 2 layers granulosa cells) and a larger number of growing follicles at each of the various stages above a diameter of 0.06 mm (Cahill et al., 1979). It has also been reported that the proportion of preantral follicles declines while the proportion of antral follicles increases during progressive oestrous cycles in each breeding season (Dufour & Guilbault, 1984).

1.2.2. Gonadotrophic Control

Granulosa cells of both large and small follicle contain FSH receptors and while binding of FSH by granulosa cells decreases with atresia, this binding is not related to follicle diameter (Carson et al., 1979). Compared to granulosa cells, ovine thecal cells

have been shown to achieve only a very low binding of FSH (Carson et al., 1979). This is probably the result of granulosa cell contamination of the thecal cell preparations used and, as in the rat (Richards, 1980) it seems probable that ovine thecal cells possess no FSH receptors. hCG, which binds in a similar manner to LH, showed high binding to antral follicle of all sizes (1-6 mm) studied by Carson et al. (1979), and like the binding of FSH to granulosa cells, LH binding to thecal cells declines with the advancement of atresia (Carson et al., 1979). LH receptors are also found in large numbers on the granulosa cells of large (> 4 mm), non-atretic antral follicles (Carson et al., 1979) and unlike FSH the overall extent of LH binding is determined by follicle diameter, the greatest responsiveness to LH being seen in the large antral follicles. It is thought that these follicles with LH receptors on the granulosa cells as well as the theca cells are the follicles that will ovulate, and it has been shown that follicles without such receptors do not luteinize and produce progesterone in response to the LH surge but become atretic (Richards & Midgley, 1976). In the sheep, England et al. (1981) found that most follicles contained either no LH receptors, or LH receptors only in thecal tissue. However, in 1 or 2 follicles per ewe LH receptors were also present on the granulosa cells. These "activated" follicles were large antral follicles which invariably possessed a high antral fluid concentration of oestradiol and were presumably at the stage during which they were able to respond to the LH surge and ovulate. This idea is supported by the observation that the number of these follicles, with LH receptors in both thecal and granulosal tissue, is the same as the ovulation rate in breeds of sheep of both low and high fecundity (Webb & England, 1982).

FSH appears to be responsible for both the induction of its own receptors and the induction of LH receptors in granulosa cells (Ireland, 1987), and it is thought that FSH is necessary for the maintenance of gonadotrophin receptors throughout folliculogenesis (Richards, 1980). Oestradiol is also thought to be involved in the generation of LH receptors on granulosa cells, as well as increasing the responsiveness of granulosa cells to FSH (Richards et al., 1976).

A third gonadotrophin, prolactin has been implicated in the control of folliculogenesis. Prolactin receptors have been found on the oocytes of small follicles in the rat (Dunaif et al., 1982), and in the sheep the number of growing preantral follicles has been shown to decrease following the use of bromocriptine to reduce prolactin levels (Cahill et al., 1984), though the number of antral follicles remained unchanged. However, most aspects of follicular growth and development appear to progress independent of prolactin (Baird & McNeilly, 1981), though the possibility of a role for this hormone cannot be excluded.

Evidence suggests that gonadotrophins act by binding to their membrane receptors and activating the adenyl cyclase enzyme system (Catt & Dufau, 1976; Marsh, 1976) resulting in cAMP production. cAMP is then thought to activate the protein kinase system resulting in the phosphorylation of the proteins involved in folliculogenesis (Catt & Dufau, 1976, Hay & Moor, 1978). cAMP probably then stimulates an increase in several aspects of steroidogenesis rather than acting at a single rate-limiting site (Marsh, 1976; Hay & Moor, 1978). In the sheep, the granulosa cells of small (1-3 mm) and large (4-6 mm) follicles have been shown to produce cAMP in response to FSH stimulation, while hCG only stimulated cAMP production from large granulosa cells (Weiss et al., 1978). This finding supports the idea that while granulosa cells possess FSH receptors at all sizes, LH receptors only appear on the granulosa cells of larger follicles. In the same study LH was found to stimulate cAMP production from the thecal cells, but not the granulosa cells, of all sizes of follicles, supporting the idea that thecal cells never develop FSH receptors.

1.2.3. Follicle Steroidogenesis

Up to a diameter of around 3mm ovine follicles contain low concentrations of oestradiol and androstenedione compared to that of testosterone (Carson et al., 1981). Above this size oestradiol production increases rapidly while testosterone concentrations fall, this change coinciding with the appearance of LH receptors on the granulosa cells (Carson et al., 1979). Atretic follicles show a similar pattern of follicular steroid content to healthy follicles below 3 mm, but fail to show an increase in oestradiol production as their size increases above this diameter (Carson et al., 1981).

The main ovarian androgens in the sheep, androstenedione and testosterone, appear to be produced entirely by the theca (Moor, 1977; Armstrong et al., 1981). Androgen release is stimulated by LH (Baird & Scaramuzzi, 1976b; Armstrong et al., 1981), apparently via cyclic AMP, the production of which is stimulated by the binding of LH to its receptors on thecal cells in a number of species including the sheep (Weiss et al., 1978). The main role of LH appears to be the stimulation of the conversion of cholesterol to pregnenolone (Marsh, 1976), stimulation of this early step in the steroidogenic pathway resulting in an increase in the production of all steroids. Granulosa cells lack the necessary enzyme activity to produce androgens, but are important in converting thecal androgens to oestrogens (Hillier, 1985).

While small antral follicles with thecal cell LH receptors have the ability to secrete a limited amount of oestradiol (Webb & Gauld, 1985), it is now well established that the majority of the oestradiol is produced by the large antral follicle(s)

in the sheep during both the luteal and follicular phase (Moor et al., 1975; Baird & Scaramuzzi, 1976b). In the sheep, as in many other species, it appears that the theca and granulosa interact in the production of oestradiol (Falck, 1959; Armstronget et al., 1981). This "2-cell theory" suggests that thecal cells produce androgen precursors under LH stimulation, and that these androgens are then converted to oestradiol in the granulosa cells by an aromatase P-450 enzyme system. This theory is supported by a large amount of evidence from a number of species. In the sheep, isolated thecal and granulosa cells are unable to secrete oestrogens, while co-cultures of the two cell types secrete oestrogens successfully (Moor, 1977; Armstrong et al., 1981), though only for a short period suggesting that full association between the two cell types was needed for successful oestrogen production. Ovine granulosa cells are 4 times more effective in converting testosterone than androstenedione (Hay & Moor, 1978), suggesting that testosterone is the more important substrate for aromatisation to oestradiol in the sheep. *In vivo* evidence for this "two-cell theory" has also been provided in the sheep by Baird (1977) who found that infusion of antiserum to testosterone resulted in a fall of over 50 % in oestrogen secretion, presumably as a result of the binding of substrate for aromatisation. In intact sheep, pulses of LH are followed by a rapid rise in the secretion of both androstenedione and oestradiol, suggesting rapid transfer of androgen precursor from the theca to the granulosa (Baird & Scaramuzzi, 1976b). It has therefore been suggested that oestradiol production takes place in the granulosa cells adjacent to basement membrane close to the capillaries supplying the theca (Baird & McNeilly, 1981), and in the rat these granulosa cells have been shown to contain more aromatising cytochrome P450 enzyme than the granulosa cells nearer the antrum (Zoller & Weiss, 1978).

Aromatase activity per follicle increases up to a follicle diameter of 3.5 mm and is then maintained at a constant level in healthy follicles (Tsonis et al, 1984a), declining in atretic follicles (Tsonis et al., 1984a; Monniaux, 1987). In the ewe FSH has been shown to increase aromatase activity in long term cultures (≥48h) (Moor, 1977; McNatty, 1982), though no such effect has been found during shorter culture periods (Monniaux, 1987). Injection of ewes with FSH has also been shown to increase aromatase activity (McNatty et al., 1985), while conversely, injection with bFF to lower FSH concentration has resulted in a reduction of aromatase activity (McNatty et al., 1985). Henderson et al. (1985) found maximum aromatase activity in cultured granulosa cells that were highly responsive to FSH in terms of cAMP production, providing indirect evidence that aromatase activity in ovine granulosa cells may be regulated by cAMP production in response to FSH. Ovine thecal cell cultures have also been shown to produce oestradiol, the production of which increased with increasing

follicle size (Armstrong et al., 1981) but was not increased by the provision of exogenous testosterone to the cultures, suggesting that the thecal oestradiol production was not the result of contamination with granulosa cells, which would have responded to the exogenous testosterone with an increase in oestradiol production.

1.2.4. Follicle Atresia

The majority of follicles do not develop to ovulation, but undergo the degenerative process of atresia. Follicle atresia is rarely seen in preantral follicles < 1 mm in diameter (Turnbull et al., 1977; Carson et al., 1979; Cahill et al., 1984) but by 1.5-2.5 mm up to two thirds of follicles may show early signs of atresia (Turnbull et al., 1977) and by the later stages of antral development up to 90% follicles may be atretic (Brand & de Jong, 1973; Turnbull et al., 1977; Cahill et al., 1979

In the early or primary stages of atresia degenerating granulosa cells with pyknotic nuclei become visible (Hay et al., 1976; Hay & Moor, 1978). As atresia progresses to the secondary phase the degree of granulosa cell degeneration increases and the inner layer of the granulosa may slough off into the antrum (Hay & Cran, 1978). This degeneration of the granulosa then spreads to the theca interna during the tertiary stage of atresia and it is not until this late stage of atresia that the oocyte is affected in the sheep, becoming necrotic as the cumulus breaks up (Hay et al., 1976). During tertiary atresia a reduction of blood flow to the inner capillary network occurs, presumably depriving the remaining granulosa cells of substrates (Hay et al., 1976), and as the process of atresia progresses the follicle eventually collapses.

Atretic follicles, however, appear to maintain a degree of viability for some time as both the thecal and granulosa layers of atretic follicles will, to a large extent, regenerate if the follicle is cultured in a suitable nutrient environment (Hay et al., 1979). Reversal of atresia *in vivo* appears to be possible in mice (Byskov, 1979) but attempts in sheep have proved largely unsuccessful (Hay et al., 1979). In the sheep and cow cumulus cells remain healthy, even after the granulosa has undergone marked degeneration (Hay & Moor, 1978), possibly as a result of preferential blood supply. As a result of this the oocytes from such atretic follicles appear to maintain full developmental capacity, as following incubation of atretic follicles under suitable hormonal conditions oocytes have gone on to produce offspring (Moor & Trounson, 1977). This idea is supported by the fact that unilateral ovariectomy 3 days before ovulation, results in ovulation by follicles that would normally have become atretic, and a normal ovulation rate is maintained (Land, 1973), a similar reduction in atresia following unilateral ovariectomy being shown by Dufour & Guilbault (1984).

Hypophysectomy results in atresia in large (> 2 mm) antral follicles in the sheep (Dufour et al., 1979). By using this technique Driancourt et al. (1987) found that large follicles took around 8 days to disappear into the ovarian stroma, while ink marking of follicles has shown that it takes about 7 days from the onset of atresia for a large follicle to disappear (Smeaton & Robertson, 1971). A rise in the incidence of atresia has been found as ewes enter anoestrus, the high level of atresia seen during the anoestrous period being accompanied by a low number of large antral follicles (Cahill et al., 1984). As the ewes return to cyclicity the level of atresia falls and the number of large antral follicles rises. These changes are presumably the result of changes in gonadotrophins, which are known to be affected by photoperiod (Lincoln & Peet, 1977). While seasonal changes in atresia seem to exist, differences in atresia do not appear to be responsible for different ovulation rates between breeds (Cahill et al., 1979).

Atretic follicles show a relatively high level of androgen production compared to that of oestradiol (Hay et al., 1979). As oestradiol production falls in the presence of high levels of androgen precursor, this is probably due to the loss of aromatase activity. It has been suggested that oestradiol production from androgens, and the responses of granulosa cells to these steroids may be important in determining whether a follicle becomes atretic, and in cattle, injection of androgens has been shown to induce atresia (Maracek et al., 1977). During atresia there is a fall in the ability of follicles to bind gonadotrophins (Carson et al., 1979), but it seems unlikely that this is a cause of atresia as this fall in binding does not become apparent until after the onset of "morphological" atresia. The removal of gonadotrophins in the sheep by hypophysectomy results in atresia in all follicles > 2mm (Dufour et al., 1979) as does the reduction in gonadotrophins resulting from GnRH immunoneutralisation (McNeilly et al., 1986) or GnRH agonist treatment (McNeilly & Fraser, 1987). This indicates that gonadotrophins have an important role in preventing atresia. Injection of PMSG has been shown to reduce the incidence of atresia as has injection of purified FSH (McNatty et al., 1985), suggesting that it is FSH rather than LH that is more important in preventing atresia. The preovulatory LH surge is thought to induce atresia in those follicles not undergoing ovulation, and this idea is supported by the fact that injection of hCG results in an increase in follicle atresia in the ewe (Turnbull et al., 1977). The idea that high levels of LH induce follicle atresia is supported by Picton (1989) who found that high amplitude LH pulses increased the incidence of atresia among follicles in FSH-stimulated GnRH agonist-treated ewes. It has also been suggested that non-steroidal components of follicular fluid may have a role in atresia (Ledwitz-Rigby, 1979), such factors possibly regulating the cellular responses to gonadotrophins.

1.3. Follicle Selection

The process of follicle selection has been divided into two events; follicle "recruitment," during which a group of follicles capable of ovulation is established, and the actual process of "selection," during which the follicle(s) destined to ovulate is selected from the group of recruited follicles and becomes "dominant", avoiding atresia.

1.3.1. Follicle Recruitment

The use of ink marking shows that follicles are recruited for ovulation from those follicles > 2mm in diameter at the time of luteal regression (Driancourt et al., 1984; Driancourt & Cahill, 1984). This idea is supported by the observation that following electrocautery of follicles at the time of luteal regression ovulation is not delayed providing some follicles > 2mm (either 2 -4 mm or > 4 mm) are left intact (Tsonis et al., 1984b). If all follicles > 2mm are destroyed a delay to ovulation of some 24h occurs (Tsonis et al., 1984b), suggesting that only follicles > 2mm can be recruited for preovulatory development. The lack of follicle growth beyond 2mm following hypophysectomy (Dulfour et al., 1979; Driancourt et al., 1987) indicates that gonadotrophins are important in follicle recruitment and preovulatory development. During seasonal anoestrus, ovulation of follicles present at the time of treatment can be induced by pulsatile injection of LH (McNeilly et al., 1982; McNatty et al., 1984), suggesting that LH is the key hormone in follicle recruitment. However, a higher proportion of ewes can be induced to ovulate by pulsatile injection of GnRH (McLeod et al., 1982 a,b) or by the administration of FSH with pulsatile LH injections (McNeilly et al., 1985). In Blackface ewes, which show a deep seasonal anoestrous, administration of LH and FSH is required if ovulation is to be induced. From these studies it appears that both LH and FSH are important in follicle recruitment and preovulatory growth, with FSH perhaps sensitising the follicle to recruitment by LH.

1.3.2. Follicle Selection

As artificially induced luteal regression at any stage of the luteal phase is followed by ovulation about 72h later (Baird, 1983) it seems unlikely that follicle selection occurs before the onset of luteal regression. Following luteal regression there is a decline in the percentage of healthy antral follicles until, by 36h, only the

preovulatory follicle(s) remain healthy (McNatty et al., 1982). It seems, therefore, that in the sheep follicle selection occurs early in the follicular phase.

One hypothesis as to how the selected follicle(s) becomes dominant over the other follicles in the ovary is that the dominant follicle(s) secretes large quantities of oestradiol and inhibin which feed back on the pituitary gland to suppress FSH secretion, starving the remaining follicles of gonadotrophic support (Baird, 1983). A second theory suggests that the dominant follicle(s) releases regulatory substances which act at an ovarian level to inhibit the development of the other follicles (Hammond, 1981).

The "FSH theory" of follicle selection assumes that folliculogenesis is a continual process, and that follicle selection occurs by chance. Following luteal regression there is an increase in LH pulse frequency which stimulates thecal androgen production. Those antral follicles with a sufficiently developed level of aromatase activity are able to convert these androgens to oestrogens which act to increase the sensitivity of the granulosa cells in these follicles to FSH. In response to this increased sensitivity to FSH the dominant follicle(s) releases increasing amounts of oestradiol into the ovarian vein exerting negative feedback on the pituitary gland and resulting in the fall in FSH concentration seen during the follicular phase (Campbell et al., 1990a). This fall in FSH, which can be quite modest (30%, Baird, 1983), deprives less advanced follicles of the gonadotrophic support required for development and they become atretic. The dominant follicle(s) survives in this FSH-deficient environment as a result of its increased responsiveness to FSH (Henderson et al., 1985), and possibly as a result of an increased blood supply (Brown & Drioncourt, 1979). Healthy follicles have been shown to contain a higher follicular fluid concentration of FSH than atretic ones (McNatty et al., 1985). However this concentration of FSH does not seem high enough to be of importance in maintaining the follicle. Injection of PMSG at this time prevents the decline in the number of healthy follicles (McNatty et al., 1982) as does infusion of FSH (Henderson et al., 1988) suggesting that this decline in FSH concentration is causally involved in the atresia of non-ovulatory follicles at this time.

According to this model multiple follicle selection can occur by one of two mechanisms (Baird, 1983). Firstly, the number of antral follicles reaching the stage of development necessary to benefit from the follicular phase rise in LH could be increased. This idea is supported by the fact that in prolific breeds of sheep, despite a lower total number of antral and preantral follicles than less prolific breeds, the number of antral follicles is increased (Cahill et al., 1979) suggesting more antral follicles would be at the right stage of growth for selection to occur at any one time. Secondly the sensitivity of the pituitary to the negative feedback by oestradiol could be reduced,

Factor	Species	Action	Reference
Luteinization Inhibitor	Pig, Cattle	Prevents LH-induced cAMP and progesterone production by granulosa cells in culture	Hammond (1981)
FSH Binding Inhibitor	Cattle, Pig,	Prevents binding of FSH to granulosa cells	Daraga & Reichert (1979) Sato et al. (1982) Sluss & Reichert (1984) Sluss et al. (1984)
LH Binding Inhibitor	Sheep, Pig	Blocks binding of LH to luteal cells	Kumari et al. (1984)
Oocyte Maturation Inhibitor	Pig, Cattle	Inhibits in vitro maturation of cumulus enclosed oocytes	Tsafriri et al. (1976) Miller & Behrman (1986)
Follicular Regulatory Protein	Pig, Cattle	Inhibits granulosa cell aromatase activity and FSH-stimulated induction of granulosa LH receptors	diZerega et al. (1984) Battin & diZerega (1985)
Follicle Growth Inhibitor	Sheep	Inhibits granulosa cell division and induces atresia	Cahill et al. (1985) Driancourt (1987)

Table 1.1.

Some putative non-steroidal factors present in follicular fluid with potential roles in the control of follicle growth and selection.

resulting in the need for higher levels of oestradiol to be secreted by the follicles before the fall in FSH could be induced. This increased resistance to oestradiol has been demonstrated in some highly fecund breeds (Land, 1976), and in such breeds a higher follicular phase concentration of oestradiol does not result in lower concentrations of FSH than are seen in less prolific breeds (Cahill, 1981). If identical gonadotrophin regimes are provided to breeds of low and high fertility following hypophysectomy, highly fertile breeds maintain a higher ovulation rate despite the same peripheral FSH concentration (Driancourt et al, 1988; Fry et al., 1988). This finding suggests that differences in ovulation rate may partly result from differences in sensitivity to gonadotrophins, or be due to factors acting within the ovary.

The second theory of follicle selection suggests that the dominant follicle(s) secretes a number of hormonal and non-hormonal factors which modulate the gonadotrophin-induced selection process (Ireland, 1987). Some of these potential regulatory factors (Table 1.1.) have been shown to have inhibitory effects on granulosa cell hormone production, supporting the idea that a dominant follicle may inhibit the development of other follicles. One of these substances, FSH binding inhibitor, has been found to be more prevalent in atretic than in healthy follicles (Sluss et al., 1984), suggesting a role for this substance in determining atresia. In primates, gonadotrophin stimulation has been found to be more effective in inducing multiple follicular development during time periods when a dominant follicle is not present (diZerega & Hodgen, 1980), adding weight to the suggestion that the dominant follicle is acting to inhibit the development of other follicles.

While the precise mechanism controlling follicle selection is not known, it seems most likely that in the sheep this process is regulated by gonadotrophins, especially FSH, while non-steroidal substances produced by the dominant follicle(s) may be important in modulating the actions of gonadotrophins and may have actions involved in inhibiting the development of other follicles.

1.4. The Structure and Physiology of Inhibin

Inhibin, by definition, is an ovarian glycoprotein hormone which suppresses pituitary gonadotrophin secretion, preferentially that of FSH. The term inhibin was first used in 1932 to describe a factor of testicular origin involved in the control of pituitary FSH secretion (McCullagh, 1932). Evidence for the existence of inhibin was not produced until the 1970s following the demonstration that FSH secretion could be suppressed by first testicular extracts (Setchell & Jacks, 1974; Franchimont et al.,

1975) and then ovarian follicular fluid (de Jong & Sharpe, 1976). It was not, however, until 1985 that the substance was first isolated (Robertson et al., 1985).

1.4.1. Forms of Inhibin

Inhibin is made up of 2 disulfide - linked dissimilar subunits, the α and the β chains, and has been isolated from a number of sources including; bovine follicular fluid (bFF: Robertson et al., 1985; Fukuda et al., 1986), porcine follicular fluid (pFF: Ling et al., 1985; Miyamoto et al., 1985) and ovine follicular fluid (oFF: Leversha et al., 1987). In these and other studies inhibin has been isolated in a number of molecular weight forms ranging from 30kDa to 120kDa (Fig. 1.2). Some of the apparently different molecular weight sizes of inhibin differ by only a single or a few kDa and may result from inherent limitations in the techniques used to determine size, but it is clear that a number of different molecular weight forms of inhibin can be isolated. Inhibin α and β subunits are coded for by separate genes (Mason et al., 1985; Forage et al., 1986) and are initially produced in large molecular weight forms as the α preproprotein and the β proprotein (Fig. 1.2). These precursor forms contain a number of proteolytic cleavage sites and it appears that cleavage at different sites results in the production of the different molecular weight forms of inhibin that have been detected, a higher degree of processing resulting in the production of smaller molecular weight forms (Fig. 1.2). For example 32, 57 and 68 kDa inhibin share a common β subunit, the size differences resulting from the degree of processing of the α subunit. In 68kDa inhibin the α subunit is made up of 3 regions; the carboxy region (αC), the amino terminal region (αN) and a further pro region. Cleavage of this pro region results in the production of 57kDa inhibin with further cleavage of the αN region resulting in the production of 32kDa inhibin, the smallest active form of inhibin isolated so far (Fig. 1.2).

As well as whole inhibin molecules, "free" α subunit proteins have also been isolated. Knight et al. (1989) have isolated 25 and 44kDa proteins in bFF which are thought to be the αC subunit of 32kDa inhibin and the αCαN subunit of 58kDa inhibin, and have demonstrated the presence of these proteins in bovine but not ovine utero - ovarian and peripheral plasma by immunoblotting. Both Robertson et al. (1989) and Sugino et al. (1989) have isolated an α chain protein comprising a 6kDa pro region disulphide linked to the αC protein of the α chain from bFF, this product being termed Pro - αC (Fig. 1.2). Robertson et al. (1989) have also isolated the "free" αN portion of the α chain resulting from the production of the Pro - αC fragment, and immunisation against this αN fragment has been shown to impair fertility in sheep

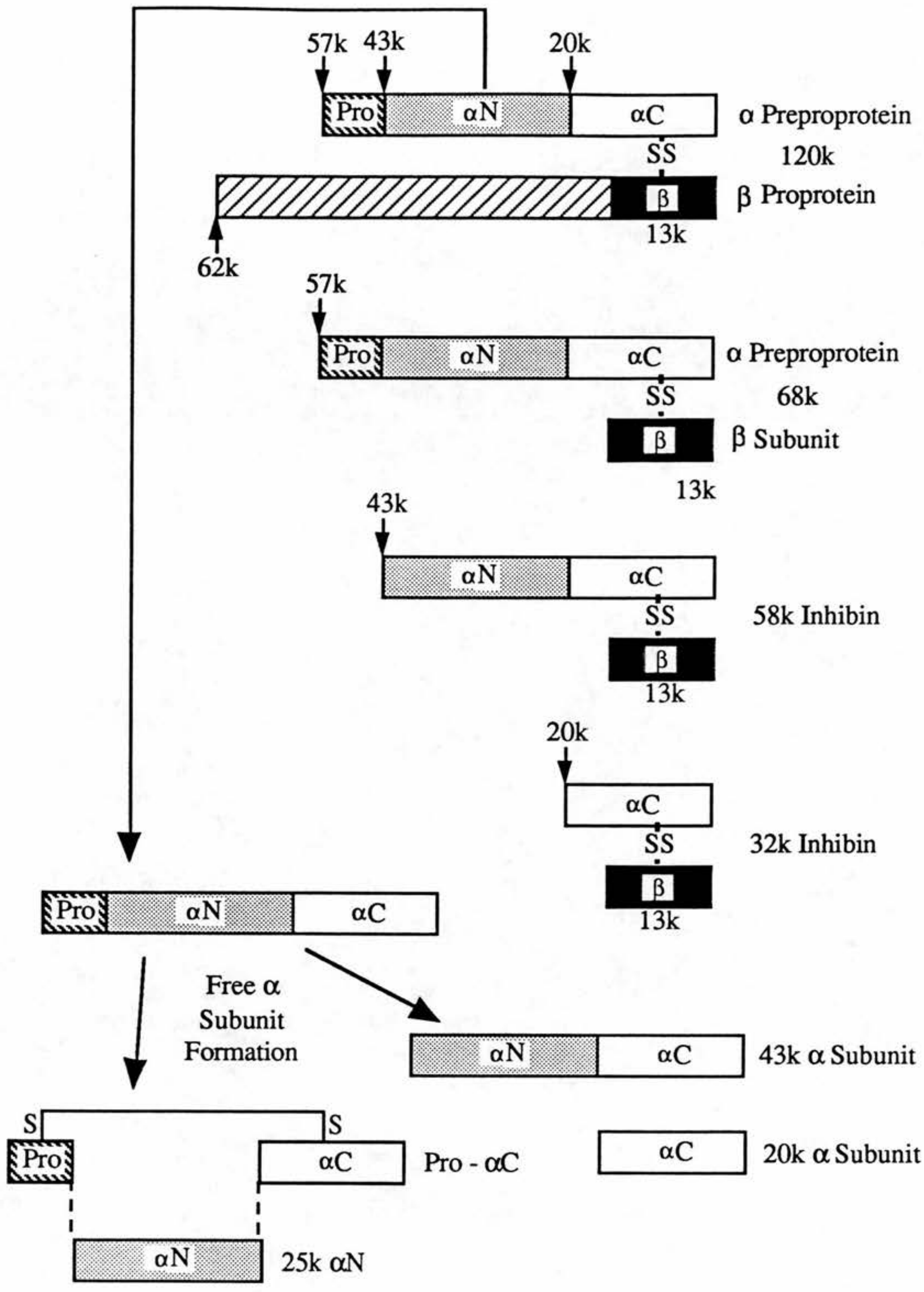

Fig. 1.2.
Schematic representation of the synthesis of some of the major forms of inhibin isolated from follicular fluid. (Based on Miyamoto et al., 1986 and Robertson et al., 1989)

(Findlay et al., 1989). While these α subunit monomers appear to be devoid of inhibin - like bioactivity (Knight et al., 1989; Robertson et al., 1989) they can be highly immunoreactive and may present a problem when trying to obtain biologically meaningful data on circulating inhibin levels.

Two forms of the β subunit of inhibin have been isolated (β_A and β_B), and mRNA for both these forms has been localised in granulosa cells (Mason et al., 1985). The resultant forms on inhibin, inhibin A and inhibin B, both have similar physiological properties (Ying, 1988). Activin, a dimer of inhibin β subunits (activin A - β_A β_A or activin AB - β_A β_B) has also been isolated from follicular fluid (Ling et al., 1986; Vale et al., 1986) though not identified in serum (Robertson et al., 1990). In contrast to inhibin, activin is able to stimulate, rather than suppress, pituitary FSH secretion (Ling et al., 1986), and may have a role in enhancing FSH stimulation of aromatase activity (Hutchinson et al., 1987).

The molecular weight forms of inhibin occurring physiologically and their biological importance in different species is still not clear. Two forms of biologically active inhibin, 30kDa and 65kDa, have so far been isolated in the sheep (Leversha et al., 1987), these forms presumably corresponding to the 32kDa form and the 58 or 68kDa form dipicted in Fig. 1.2. While "free" αC and αCαN α chain peptides have not been found in ovine plasma (Knight et al., 1989), the quantities of αN and Pro - αC peptides remain to be determined.

1.4.2. Measurement of Inhibin

Initial measurements of inhibin activity were made using a variety of bioassays. Early bioassays relied on *in vivo* criteria such as reduction of serum FSH levels by inhibin (Nandini et al., 1976) or the inhibition of hCG - induced ovarian and uterine weight gain (Ramasharma et al., 1979). Such assays were able to detect inhibin but were not sufficiently sensitive to make accurate quantitative measurements. The next development was the use of bioassays utilising *in vitro* rat pituitary cell cultures. Bioassays for inhibin were developed using pituitary cell FSH content (Scott et al., 1980), inhibition of GnRH stimulated FSH release (Eddie et al., 1979) and inhibition of basal FSH secretion (de Jong et al., 1979) as end points. These *in vitro* bioassays were more sensitive than earlier *in vivo* assays and were able to measure high concentrations of inhibin such as those found in follicular fluid. With the advent of more sensitive bioassays for inhibin, such as that described by Tsonis et al. (1986), which uses suppression of ovine pituitary cell FSH production as an end point, it has

now become possible to measure plasma concentrations of bioactive inhibin in certain physiological situations.

The potential presence of other substances which affect FSH secretion such as activin or FSH suppressing protein (follistatin), which has been isolated from bFF and pFF (Ying, 1988) and shown to suppress FSH secretion by pituitary cell cultures, presents an obvious problem in obtaining true values of inhibin bioactivity. Before being measured in inhibin bioassays samples are usually treated with charcoal to remove steroids which would suppress FSH secretion. This treatment may affect the inhibin present in the samples adding further to the problems in obtaining true estimates of inhibin bioactivity.

Once inhibin had been isolated and purified it became possible to develop radioimmunoassays for inhibin. Two types of assay have been developed based on antisera raised either to native inhibin (Mclachlan et al., 1986; Hasegawa et al., 1987; Robertson et al., 1988) or to synthetic peptides of the α subunit (Rivier et al., 1986; McNeilly et al., 1989; Knight et al., 1989). Both types of radioimmunoassay appear to have been successfully applied to the measurement of inhibin in the serum of a number of species including the sheep (McNeilly et al., 1989; Campbell et al., 1990a; Findlay et al., 1990). While both types of inhibin radioimmunoassay appear to show little or no cross reactivity with inhibin - related proteins such as TGFβ, they do cross react with some of the forms of biologically inactive "free" α subunit peptide isolated from follicular fluid (Robertson, 1990). At present detailed information on the levels of these α subunit fragments in plasma is not available and so, for the moment, the biological significance of measurements of immunoreactive inhibin concentrations should be viewed with some caution.

1.4.3. Ovarian Production of Inhibin

Inhibin is produced by the granulosa cells of all species studied so far including cattle (Henderson & Franchimont, 1981) and sheep (Campbell, 1989), and accumulates in the follicular fluid. Following cautery of all visible large antral follicles in the ovary the secretion rate of inhibin into ovarian effluent lymph (Findlay et al., 1986) and ovarian venous plasma (Findlay et al., 1990) declines, providing evidence that large antral follicles are a source of ovarian inhibin secretion. The role of the follicles in inhibin production was supported by the studies of Rodgers et al. (1989) who demonstrated the presence of mRNA for both the α and β_A inhibin subunits in the follicles of sheep and cattle, and by the *in situ* hybridisation studies of Torney et al.

(1989) who have localised mRNA for these two inhibin subunits in the granulosa cells of bovine follicle.

Inhibin production, assessed by the presence of inhibin in the antral fluid, has been shown in follicles of > 1.4mm diameter (Tsonis et al., 1983), with the follicular fluid of larger follicle containing more inhibin. Inhibin production by bovine granulosa cells has been shown to increase with follicle size (Henderson et al., 1984a), suggesting that larger follicle are a more important source of inhibin. Torney et al. (1989) found mRNA for the inhibin α subunit in bovine follicles as small as 0.36mm diameter but only found mRNA for both α and β_A subunits in follicle > 0.8mm. In agreement with the studies of Tsonis et al. (1983) and Henderson et al. (1984a), Torney et al. found an increase in mRNA for α and β_A inhibin subunits with follicle diameter. In their studies Torney et al. (1989) also found mRNA for the β_A inhibin subunit in thecal cells of follicles > 0.8mm suggesting that the theca may also have some role in inhibin production. As well as the apparent association with follicle diameter, inhibin production has been shown to decline with morphologically (Henderson et al., 1984a) and biochemically (Tsonis et al., 1983) determined atresia.

In humans there is an increase in circulating inhibin concentrations during the luteal phase (McLachlan et al., 1987), suggesting that the corpus luteum may be an important source of inhibin. This idea is supported by the fact that human granulosa cells allowed to luteinize in culture secrete inhibin (Tsonis et al., 1987). Furthermore, in both the human (Davis et al., 1987) and the rat (Davis et al., 1986) mRNA for inhibin has been detected in luteal tisue. In the sheep the secretion rate of bioactive inhibin has been shown to be higher from ovaries containing luteal tissue, and to decline following luteal regression (Tsonis et al., 1988a) suggesting that the corpus luteum may also secrete inhibin in this species. Attempts to localise mRNA for inhibin in the corpus luteum have failed in sheep and in cattle (Rodgers et al., 1989; Torney et al., 1989), and cultured bovine luteal cells have failed to secrete inhibin (Henderson et al., 1981). The potential of the corpus luteum as a source of inhibin therefore remains unresolved in the sheep.

1.4.4. The Control of Inhibin Production

In the rat, FSH stimulates the production of inhibin both *in vivo* (Lee et al., 1981) and *in vitro* (Bicsak et al., 1986), and LH has also been shown to stimulate inhibin production *in vitro*. In sheep, however, the evidence for gonadotrophic stimulation of inhibin production is less clear. Inhibin secretion from the ovary is unaffected by endogenous (McNeilly & Baird, 1989) or exogenous (Campbell et al.,

1989; Scaramuzi et al., 1989) pulses of LH, and inhibin production by cultures of ovine and bovine granulosa cells also appears to be unresponsive to stimulation by LH (Henderson et al., 1984a; Campbell, 1989). FSH was shown to cause a small but significant stimulation of inhibin production by cultures of bovine granulosa cells from large healthy, but not atretic, follicles (Henderson et al., 1984a). In the ewe, continuous infusion of FSH for 48h after luteal regression results in an increase in inhibin production (McNeilly et al., 1989), but this increase may have resulted from the stimulation of the development of more follicles as acute stimulation with FSH appears to have no effect on the ovarian secretion rate of inhibin (Scaramuzzi et al., 1989), and stimulation of ovine granulosa cell cultures with FSH does not result in an increase in inhibin production (Campbell, 1989). It appears, therefore, that while FSH may have a direct role in the control of inhibin production by ovine and bovine granulosa cells, this role is by no means clear. Luteinizing hormone, on the other hand, does not appear to have any role in the control of inhibin production in the sheep.

In bovine granulosa cell cultures, testosterone and androstenedione stimulate inhibin production (Henderson and Franchimont, 1981; Henderson et al., 1984a) while oestradiol has no effect (Henderson and Franchimont, 1981). This would suggest a role for androgens in the control of inhibin production. Henderson and Franchimont (1981) found that in the absense of serum, cultured bovine granulosa cells produced no inhibin, and suggested that the factor in serum responsible was not FSH or LH as neither gonadotrophin could stimulate inhibin production in the absence of serum. Campbell (1989) found that insulin stimulated inhibin production from ovine granulosa cells, and it is possible that insulin or insulin like growth factor may play a role in controlling granulosa cell inhibin production.

1.4.5. Inhibin Secretion During the Oestrous Cycle

The secretion of inhibin is pulsatile during both the follicular phase (McNeilly & Baird, 1989; Murray et al., 1989; Campbell et al., 1990b) and the luteal phase (Campbell et al., 1990b). These pulses, which occur at intervals of approximately 1h at all stages of the cycle (Campbell et al., 1990), do not appear to be related to pulses of LH, as is the case for ovarian steroids, and their aetiology remains unknown. Bioactive inhibin levels have been shown to decline following luteal regression (Tsonis et al., 1988a) but this finding has not been confirmed by radioimmunoassays. Using an assay against a peptide fragment of the α chain or inhibin McNeilly et al. (1989) found no change in the secretion rate of inhibin following luteal regression, while Campbell et al. (1990) found a significant but inconsistent rise in inhibin secretion at this time. Using

an assay based on entire 31kDa bovine inhibin Findlay et al. (1990) also found a rise in inhibin concentration at this time. Following the LH surge Campbell et al. (1990a) found a rise in the secretion rate of inhibin resulting in a high secretion rate at the time of the second FSH peak, while Findlay et al. (1990) found a decline in peripheral inhibin concentration. These differences are hard to reconcile, bearing in mind that the two assays have been shown to give comparable results (Campbell et al., 1990a), but may be due to breed differences or to an insufficiently frequent sampling interval.

1.4.6. The Physiological Role of Inhibin

The role of oestradiol in the control of FSH secretion in the sheep is now beyond doubt, but many aspects of the part inhibin plays in this process remain to be fully determined. The rise seen in FSH concentration following ovariectomy can only be partially prevented by treatment with "physiological" amounts of oestradiol (Goodman et al., 1981b; Martin et al., 1988), while inhibin and oestradiol together are able to maintain the concentration of FSH (Martin et al., 1988). This suggests that inhibin and oestradiol act together in the control of FSH secretion.

It is now well established that inhibin in steroid free follicular fluid suppresses FSH secretion in both intact (McNeilly, 1984; Wallace & McNeilly, 1985) and chronically ovariectomised (Cummins et al., 1983; Findlay et al., 1985) ewes, and purified inhibin has now been shown to suppress FSH secretion in ovariectomised ewes (Findlay et al., 1987). Inhibin from follicular fluid will also prevent the post castration rise in FSH seen in acutely ovariectomised ewes if supplied in sufficiently large quantities (Martin et al., 1986, 1987) indicating the potential of inhibin as a regulator of FSH secretion.

The action of inhibin appears to be exerted, at least in part, on the pituitary as inhibin in steroid free follicular fluid and purified inhibin both suppress FSH release from cultured ovine pituitary cells (Tsonis et al., 1986; Findlay & Clarke, 1987). Clarke et al. (1986) have shown that inhibin in oFF was able to suppress pituitary FSH production to a large degree in ovariectomised ewes with hypothalamo - pituitary disconnections which were pulsed with exogenous GnRH, indicating that inhibin does not exert its negative feedback action at the hypothalamus. This idea is supported by the fact that inhibin in steroid free bFF has been shown to suppress pituitary FSH secretion in ewes both before and after desensitization of the pituitary to GnRH by chronic GnRH agonist treatment (Knight et al., 1990).

Following cessation of treatment with inhibin, a "rebound" in FSH secretion occurs (Miller et al., 1982; McNeilly, 1984), the release of FSH during this rebound

period ranging from 100 - 500% of the amount not released during the period of suppression (McNeilly & Wallace, 1987). This rebound effect is not seen in ovariectomised ewes (Findlay & Clarke, 1987), suggesting that it is associated with the ovaries. This effect could be due to a reduction in oestradiol and/or inhibin secretion from ovarian follicles resulting from the period of FSH suppression, or may be due to a factor in follicular fluid, other than inhibin, which directly suppresses follicular development and hormone production (Baird et al., 1990; Law et al., 1990).

Treatment with large amounts of inhibin in follicular fluid has been shown to partially inhibit the rise in LH concentration seen following ovariectomy (Martin et al., 1986), and to cause a reduction in LH pulse amplitude and/or frequency (Findlay et al., 1987; Martin et al., 1986, 1987; Clarke et al., 1986). However, other studies using large doses of inhibin have shown no effect on LH (McNeilly, 1984, 1985) or even an increase in LH pulse amplitude (Wallace & McNeilly, 1986). Studies in which inhibin was administered in amounts corresponding to "normal" ovarian output have shown no effects on LH secretion (Martin et al., 1987, 1988), suggesting that the effects seen in some of the studies using larger doses of inhibin were pharmacological effects. Inhibin from oFF has no effect on basal LH production by cultured ovine pituitary cells (Tsonis et al., 1986) and so it would appear that in the sheep inhibin is acting as a selective suppressor of FSH secretion, though the possibility of a role for inhibin in the control of LH secretion cannot be completely excluded.

Active immunisation against oestradiol results in a marked elevation in the peripheral concentrations of FSH and LH and causes multiple follicular development (Pant & Rawlings, 1973; Martenz et al., 1979), though ovulation does not occur due to the lack of a preovulatory gonadotrophin surge resulting from the removal of oestradiol positive feedback. Active immunisation of ewes against inhibin - enriched fractions of bFF has been shown to result in an increase in ovulation rate (Henderson et al., 1984b; Cummins et al., 1986), as has active immunisation of cattle against inhibin purified from oFF (Price et al., 1987). Ovulation rate has also been shown to increase following immunisation of ewes against a fragment of the inhibin α subunit produced by recombinant DNA techniques (Forage et al., 1987) suggesting that the effect seen in the studies using inhibin purified from follicular fluid were due to antibodies to inhibin and not to some other factor in the immunogen preparation. In these active inhibin immunisation studies the expected rise in plasma FSH concentration has not been detected or has been small and variable. Passive administration of antibodies to inhibin in ovariectomised ewes prevents the suppression of FSH caused by treatment with bFF (Al-Obaidi et al., 1986), and inhibin antibodies have been shown to neutralise the suppressive effects of inhibin preparations on FSH release by pituitary cell cultures

(van Dijk et al., 1986). It seems probable, therefore, that the effect of active immunisation against inhibin on ovulation rate is mediated through the control of FSH secretion, and it is possible that more intensive techniques are required to successfully detect these changes in FSH. It is, however, also possible that inhibin has direct effects on the ovary. Active immunisation of ewes against the αN "free" α chain peptide, which does not appear to possess any inhibin - like biological activity in regard to FSH secretion, has been shown to reduce fertility (Findlay et al., 1989), supporting the idea that inhibin may have intra - ovarian roles. Whatever the mechanism governing these changes in ovulation rate after immunisation against inhibin, these studies do demonstrate that inhibin has a physiological function in the ewe.

Chapter 2

Materials & Methods

2.1. Experimental Animals

Two breeds of ewe were used in the experiments described in this thesis, Scottish Blackface ewes and Finn x Merino ewes. The Blackface ewes were mature ewes bought each autumn from the Macaulay Land Use & Research Institute Farm, Sourhope, Berwick, Roxboroughshire, and had been culled on the basis of age or loss of teeth, all animals being of sound health and reproductive status. The mean weight of these animals was 58.2±0.6 kg (n = 98). Prior to experiments animals were kept either in small paddocks outside, or inside in large communal pens and fed on hay and cereal based ewe nuts (Dalgety Feeds). During experimental procedures animals were housed inside in small individual pens or metabolism crates with constant access to water, and were fed hay and ewe nuts twice daily. Experiments on these animals were carried out during the breeding season between October and February, the breeding season for this breed lasting from September to March.

The Finn x Merino ewes used in the dose trial in Chapter 6 were mature 2 - 3 year old ewes (mean weight 42.3±2.2 kg; n = 6), while the ewes used in Chapter 9 were mature 7 - 9 year old ewes (mean weight = 50.3±1.4 kg; n = 12) in which the left ovary and its vascular pedicle had been transplanted to a site in the neck at least 5 years previously to allow direct sampling of ovarian venous blood in the conscious animal (Goding et al., 1967). At surgery the right ovary is removed, and the left ovary along with its vein and artery dissected out. The left ovary is then located in a surgically produced fold of skin in the neck and the ovarian vein and artery connected to the jugular vein and carotid artery, which had both been previously located in a surgically produced skin loop. The layout of the ovary and its vasculature in this model is shown in Fig 2.1. These ewes were experimented on during the breeding season in February, the breeding season in this breed lasting till late March.

2.2. Oestrus Synchronisation

Oestrous cycles were initially synchronised by the removal of medroxyprogesterone acetate intra vaginal sponges (60mg/sponge; Upjohn Animal Health Division, Crawley, Sussex, U.K.) which had been left in place for 13 days. Ewes showed oestrus within 72h as indicated by colour marking by a vasectomised ram with a coloured raddle harness. Closer synchronisation was then achieved by

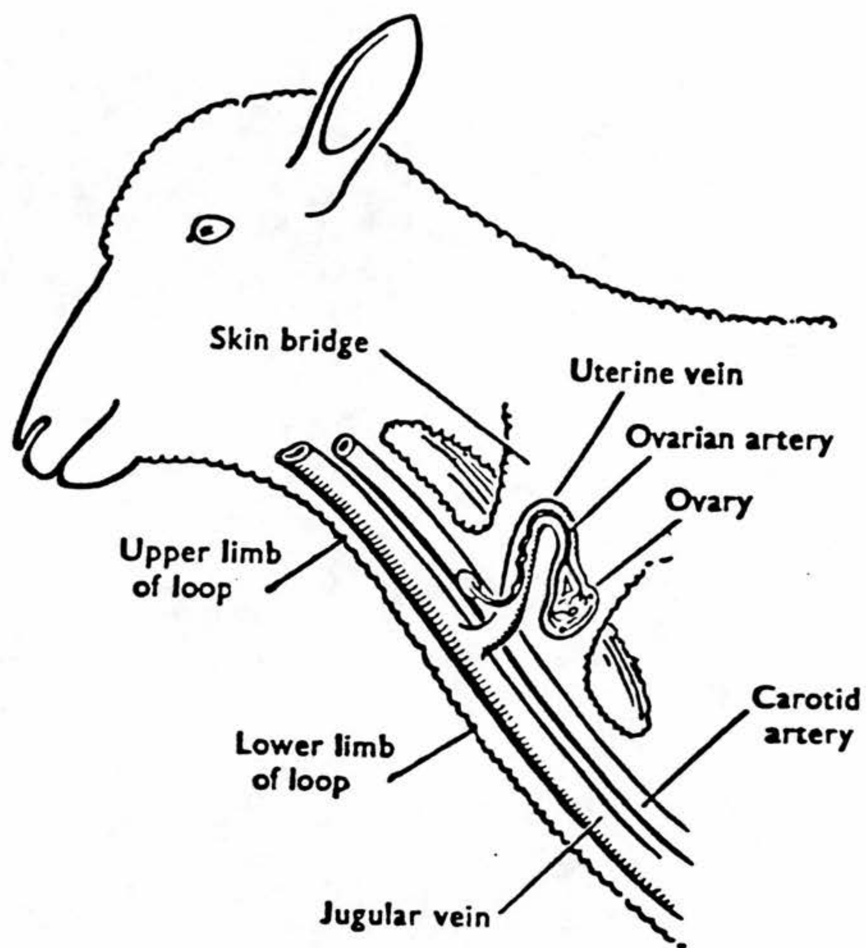

Fig. 2.1.

Diagram of the ovarian transplant preparation showing the location of the ovary in the neck and the arrangement of the blood vessels in a skin loop. (Based on Goding et al., 1967).

giving an injection of 100μg Cloprostenol, a potent analogue of prostaglandin F2α (Estrumate; Cooper's Animal Health, Crewe, Cheshire, U.K.) on day 10 of the resulting cycle, the second oestrus occurring about 48h after the cloprostenol injection.

2.3. Jugular Venous Blood Sample Collection

Less frequent blood samples (e.g. twice daily) were collected using 7ml heparinised evacuated tubes (Vacutainers; Becton Dickinson UK Ltd., Oxford) and two way needles. Where more frequent sampling was required the jugular vein was chronically cannulated using a plastic cannula (Braunula, 14 G; Dunlop Veterinary Supplies, Dumfries, U.K.), which was stitched into the neck for security. The cannula was then connected to a length of polythene manometer tubing (Portex Ltd, Kent, U.K.) with a three way tap attached (Viggo Products, Helstingborg, Sweden; sterilised by BOC Health Care). To minimise the effects of stress animals were cannulated several hours before the start of blood sampling was started. After each sample was collected the cannula and tube were flushed with sterile 0.9% (w/v) saline containing 20 i.u. heparin/ml (Leo Laboratories, Aylesbury, Bucks., U.K) to prevent clotting. After withdrawal by syringe (Plastipax; Becton Dickinson UK Ltd., Oxford) blood samples were placed in 5ml or 7ml plastic tubes (Sarstedt, Leicester, U.K.) containing heparinised beads (Sarstedt, Leicester, U.K.) and centrifuged at 1000g for 20 mins. The plasma was then decanted into 3ml or 5ml storage tubes and frozen at - 20°C prior to assay.

2.4. Ovarian Transplant Blood Sampling

In these ewes the left ovary and its vascular pedicle had been transplanted to a site in the neck (Fig. 2.1), as described in section 2.1. A length of Silastic tubing (Dow Corning Silastic Medical Grade Elastomer; Hospital Management Supplies, Carnegie Rd, Glasgow, U.K.) was inserted into the jugular vein close to the head through the bore of a suitably sized needle, and passed down the vein until its tip lay opposite the site of anastomosis of the ovarian vein. To allow sample collection a pneumatic cuff was placed around the upper limb of the skin loop and inflated to 100 - 120 mm Hg to temporarily stem jugular blood flow. Ovarian blood was then collected by free fall following manual occlusion of the jugular vein in the lower limb of the loop. This technique ensured that the blood flowing down the ovarian vein passed into the cannula with minimal dilution by jugular venous blood. To allow the ovarian blood flow rate to

be calculated timed samples of 7.5 ml of ovarian blood were collected. Ovarian blood flow measured by this technique is an over-estimate due to a contribution from the skin of the loop, but such skin contributions do not alter the calculated ovarian hormone secretion rate. A small quantity of each blood sample was used to determine the haematoctrit. This was done by filling heparinised capillary tubes (Hawksley & Son, Lancing, Sussex, U.K.) with blood, sealing one end of the tube, and then centrifuging for 10 min in a Minor™ centrifuge (MSE Crawley, Sussex, U.K.). The percentage of red blood cells was then read using a Micro-Haematocrit reader (MSE Crawley, Sussex, U.K.). The actual secretion rate of a particular hormone was then calculated from the ovarian venous plasma hormone concentration, the flow rate and the haematocrit, and was corrected for peripheral hormone concentration (Collet et al., 1973).

2.5. Oestradiol Antibody Generation

2.5.1. Conjugate Preparation

To produce the hormone antigen for immunisation 10 mg 6-ketoestradiol 6-O-carboxy methyl oxime (Sigma, Fancy Road, Poole, Dorset, England) was conjugated to a rabbit serum albumin (Sigma) carrier protein using a mixed anhydride reaction (Erlanger, Borek, Beiser & Lieberman, 1957), and the conjugate made up to 10 ml in sterile saline.

2.5.2. Immunisation Procedure

An emulsion of the conjugate was prepared with Freund's complete adjuvant (Sigma), using 1ml of the antigen, 1ml saline and 6 ml adjuvant, and 4 doses of 0.5 ml of this preparation were immediately administered to each of 3 ovariectomized Finn x Merino. These injections of antigen emulsion were given subcutaneously at sites at the front and rear of the flank on either side of each ewe. Booster immunisations were given at 1 month and 2 months after the initial immunisation by an identical procedure. Blood samples were collected by jugular venepuncture immediately prior to immunisation and at various times before and after subsequent booster immunisations to allow plasma antibody titres to be monitored. The titres achieved with time in the ewe from which antiserum was subsequently used for passive immunisation (Ewe 2G14) are shown in Fig 2.2. In this ewe exsanguination was performed 12 days after the final booster immunization and the plasma from this final bleed used for the passive

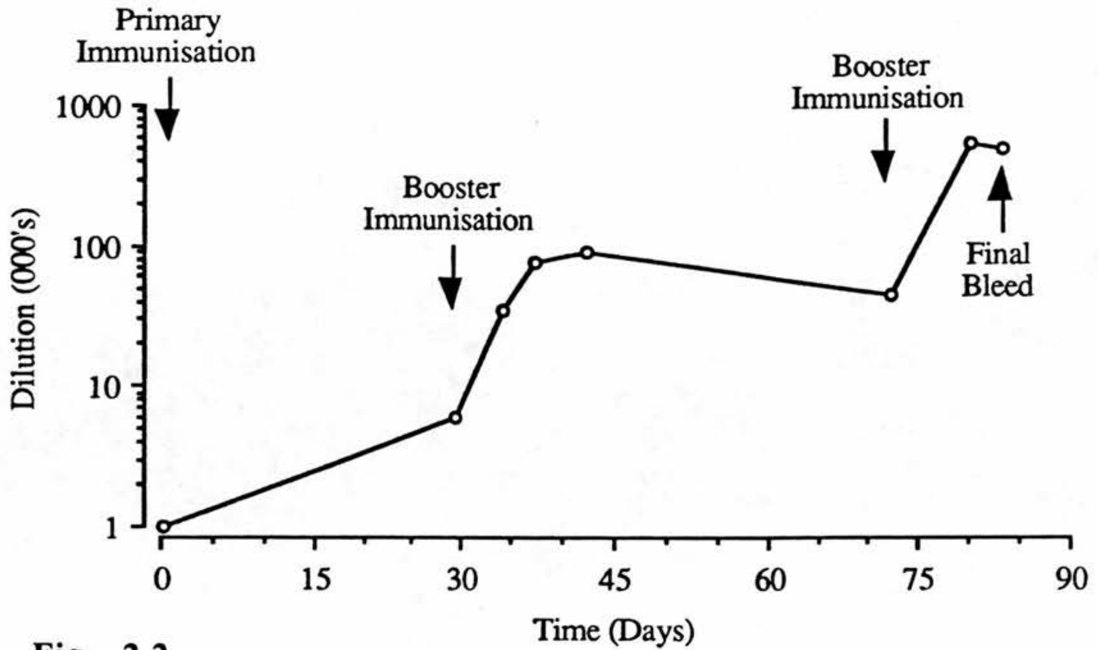

Fig. 2.2.
The change in plasma titre (assessed at 50% binding of ^{125}I - labelled oestradiol) in ewe 2G14 at various time following primary immunisation at time 0 and booster immunisations at 29 days and 72 days against an oestradiol - rabbit serum albumin conjugate.

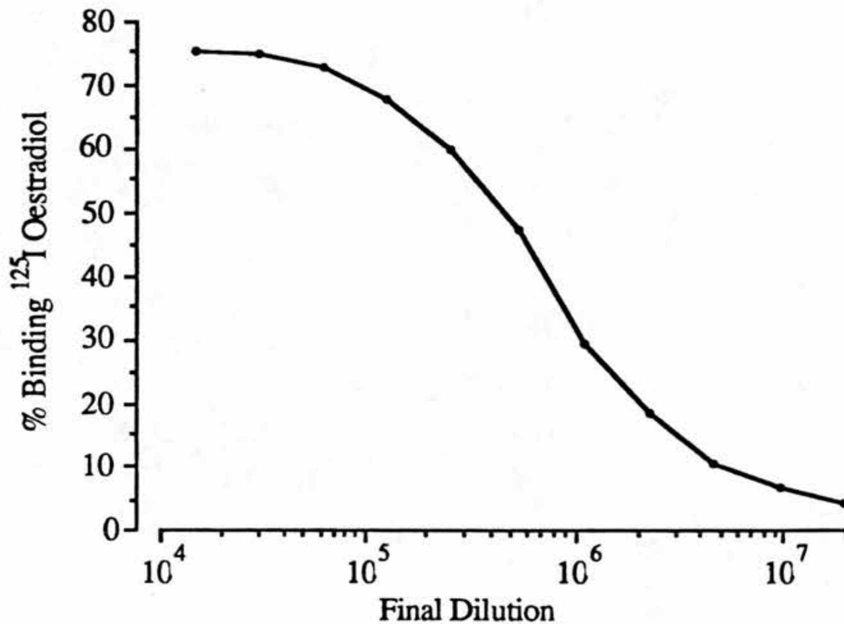

Fig. 2.3.
Percentage binding of ^{125}I - labelled oestradiol by dilutions of the plasma from the final bleed of a ewe (2G14) immunised against an oestradiol - rabbit serum albumin conjugate.

immunisation studies. During exsanguination blood was collected into heparinised 50ml tubes via a 14 gauge needle inserted into the jugular vein and centrifuged at 2000g for 30 min. Plasma was then poured off, pooled and then aliquotted and frozen at - 20 °C until required.

2.5.3. Measurement of Antibody Titre and Specificity

Oestradiol titre in the plasma of immunized ewes was determined by measuring the binding of ^{125}I - labeled oestradiol by several dilutions of plasma. Incubations were carried out in 0.075mol phosphate-buffered 0.9% (w/v) saline/l (pH 7.2) containing 1% (w/v) gelatin and 0.01% (w/v) thiomersalate (PGBS). The ^{125}I - labelled oestradiol was made up to 15 000 cpm in the buffer and 0.1ml tracer, 0.1ml plasma dilution and 0.1ml buffer (final volume 0.3ml) incubated in 10 x 75mm glass tubes overnight at 4°C. Bound tracer was then separated by adding 0.5ml Dextran Charcoal (0.125% dextran, 1.25% charcoal in PGBS) and centrifuging at 2000g for 15 min. The supernatant (bound fraction) was then counted and binding expressed as a percentage of the total counts added. A plot of plasma dilution against percentage binding was then plotted, and the titre defined as the dilution of plasma giving 50% binding. A titre curve of the final bleed from ewe 2G14 is shown in Fig 2.3. The titre of the plasma from this bleed was expressed as 50% binding of total tracer added was 1:500 000.

Specificity of the antibody was determined by incubating several dilutions of the hormone whose cross - reactivity was being tested with a fixed dilution of the antibody (1:200 000) which gave 65% binding. The inhibition curve was then compared to the inhibition curve for oestradiol and specificity estimated on a mass basis (Scaramuzzi et al., 1975). The cross-reactivities, determined at a final antibody dilution of 1:200000, were 100% for oestradiol-17b, 14% for oestrone, and < 0.05% for progesterone, testosterone and androstenedione.

2.6. Inhibin Antibody Production

The anti-inhibin antiserum used in this study was raised in an ovariectomized Scottish Blackface ewe (McNeilly, Swanston, Crow et al. 1989) to a synthetic peptide of the 1-26 amino acid sequence of the N-terminus of the a chain of 32 kDa porcine (p) inhibin (Rivier et al, 1986) conjugated to ovalbumin. The final titre of this antibody, assessed at 50% binding of ^{125}I-labeled p1-26a peptide was 1: 40000. Prior to its use in passive immunisation studies this antibody was shown to effectively neutralise the inhibin activity in oFF *in vitro* (See Chapter 6).

The inhibin titre was determined by measuring the binding of ^{125}I-labeled p1-26a inhibin peptide by a series of dilutions of the antibody. Incubations were carried out in 0.075M phosphate buffered 0.9% saline (pH 7.4) containing 1% bovine serum albumin and 0.01% thiomersalate. The ^{125}I - labeled p1-26a inhibin peptide was made up to 15 000 cpm in the buffer and 0.1ml tracer, 0.1ml plasma dilution, and 0.1ml buffer (final volume 0.3ml) incubated in 10mm x 65mm plastic tubes overnight at 4°C. Bound tracer was then separated by adding 1ml of 15% PEG + 0.1% Triton-X in 0.9% saline and centrifuging at 3000rpm for 30 minutes. The precipitate (bound fraction) was then counted and binding expressed as a percentage of the total counts added.

2.7. Ovine Follicular Fluid Collection

The ovine follicular fluid (oFF) used in the experiments in chapters 5 and 9 was from a single pool of follicular fluid collected from ovaries obtained from a local abattoir. All visible follicles were aspirated and the fluid frozen at - 20°C. The follicular fluid was treated with charcoal to remove steroids. This was done by incubating the follicular fluid with 5mg charcoal/ml at room temperature and then centrifuging at 1000g for 30 min at 4°C to remove the charcoal.To remove any residual steroids the fluid was then passad through C18 Sep-Pak cartridges (Waters Associates, Milford, MA, U.S.A.)

2.8. Inhibin Assays

2.8.1 Inhibin Immunoassay

Samples of plasma and culture medium were assayed by immunoassay for inhibin using an assay developed by McNeilly et al. (1989). The antibody used in this assay (R150) was raised in a rabbit to a synthetic peptide fragment of the 1 - 26 amino acid sequence of the N - terminus of the α chain of 32 KDa porcine inhibin (Rivier et al., 1986) conjugated to ovalbumin. This antibody was used at a final dilution of 1: 75000, and the 1 - 26 α peptide fragment was labelled with ^{125}I iodine using chloramine - T, for use as tracer. The inhibin peptide fragment was also used as standard in the range 39 - 10000 pg/ml. When assaying plasma samples (up to 300µl/tube) an equal volume of plasma from an ovariectomised ewe was added to the NSB, B_O and standard tubes to offset a small (5 - 10%) degree of suppression of binding caused by the presence of plasma. Prior to use batches of this ovariectomised plasma were checked to ensure that they did not displace inhibin tracer in the assay. The

sensitivity of the plasma assay ranged from 20 - 40 pg/ml, and intra and inter assay coefficients of variation were 7% and 9.5%. The sensitivity of the culture medium assay was 50 pg/ml and the intra and inter assay coefficients of variation 9% and 13%. The protocol for the inhibin immunoassay can be found in the appendix (Section 10.1.6).

2.8.2 Inhibin Bioassay

Inhibin bioactivity was measured using a previously described bioassay (Tsonis et al., 1986). Briefly cells from ovine pituitaries were dispersed using trypsin digestion and diluted to a final concentration of 200000 cells per 50μl supplemented Dulbecco's Modified Eagle's Medium (DMEM; Flow Laboratories, Rickmansworth, Herts) containing 10% lamb serum, 2.5% fetal bovine serum, 10mmol NaHCO3/l, 2mmol glutamine/l (Sigma), and penicillin (50i.u./ml) and streptomycin (50μg/ml; Flow Laboratories). Viability, measured by Trypan Blue exclusion, was 90-95%. Aliquots of 50μl of cells (200000) were plated out with 550μl modified DMEM into individual wells of plastic culture plates (Costar; Flow Laboratories) and preincubated for 48h. After 48h the medium was removed and replaced with inhibin standard (oRTF; 50 μl) or sample (50 - 250 μl), and supplemented medium to a final volume of 600μl, and incubated for a further 48 h. Medium was then collected and frozen at - 20 °C until assayed for FSH.

The standard used was a stable lyophilised preparation of oRTF with an arbitary potency of 1U/mg (Eddie et al., 1979). A quality control of steroid stripped oFF (1083 oFF; Tsonis et al., 1986) was included in each assay. The standard and quality control were assayed in quadruplicate at 5 doses, while the samples were assayed in triplicate using 3 doses ranging between 25 - 250 μl/well. An FSH dose inhibition curve, expressed as a percentage inhibition of control wells with no exposure to inhibin, was produced for each sample and regression analysis was performed using a computer program (Scott, Burger & Quigg, 1980). The slope, index of precision (l), and significance of regression (Finney's G; F statistic for significance/F statistic for regression) calculated (Finney, 1964), and samples were then compared with a reference standard and the relative potency calculated, providing there was no significant deviation from parallelism or linearity ($p > 0.05$; Borth, 1976). The minimum sensitivity of the bioassay was 0.05 -0.1U/ml, the index of precission (l) 5.2% and the significance of regression (G) < 0.02. The inter assay coefficient of variation was 14.9%.

2.9. Gonadotrophin Assays

2.9.1. M91/1 FSH Assay

This specific double antibody radioaimmunoassay, described by McNeilly et al. (1976), was used to measure FSH concentrations in culture medium from the inhibin bioassay and plasma samples from the experiment described in chapter 5. The assay uses a rabbit anti - human FSH antiserum M91/1 at a final dilution of 1:30000 and ^{125}I - labelled ovine FSH (Jutisz, FSH 181 - 2) as tracer. NIH - oFSH - S9 was used as standard in the range 4 - 1000 ng/ml. The sensitivity of the culture medium assay (90% B/B_o) was 16ng NIH - oFSH - S9/ml and the intra and inter assay coefficients of variation 8.2% and 11.4%. The sensitivity of the plasma assay was 40ng NIH-oFSH-S9/ml and the intra assay coefficient of variation for 3 quality control pools 4.2%: all samples were included in a single assay. The protocol for both the plasma and the bioassay medium assay can be found in the appendix (Section 10.1.3).

2.9.2. NIAMMD - I - 1 FSH Assay

In all other experiments FSH was measured in plasma samples using the assay described by McNeilly et al. (1976) with rabbit anti- ovine FSH NIAMDD - 1 as first antibody at a final dilution of 1:96000. This assay was run with a more highly purified standard, oFSH RP1, used in the range 0.2 - 30 ng/ml, with ^{125}I - labelled NIAMDD oFSH - I - 1- as tracer. A comparison of the standard curves for the two assays is shown in Fig. 2.4. The sensitivity of this assay was in the range 0.2 - 0.3ng NIH-oFSH-RP1/ml and the intra and inter assay coefficients of variation for 3 quality control pools 7.2% and 10.1%. The protocol for this assay can be found in the appendix (Section 10.1.4).

2.9.3. LH Assay

LH was measured in plasma samples using the assay described by McNeilly et al. (1976) with rabbit anti - oLH R29 as first antibody at a final dilution of 1:600000. The assay was initially run with NIH - oLH - S18 as standard used in the range 0.2 - 50 ng/ml, and with ^{125}I - labelled oLH LER - 1056 - C2 as tracer. The sensitivity of this assay was 0.2ng NIH - oLH - S18/ml and the intra and inter assay coefficients of variation for 5 quality control pools 9.4% and 12.3%.

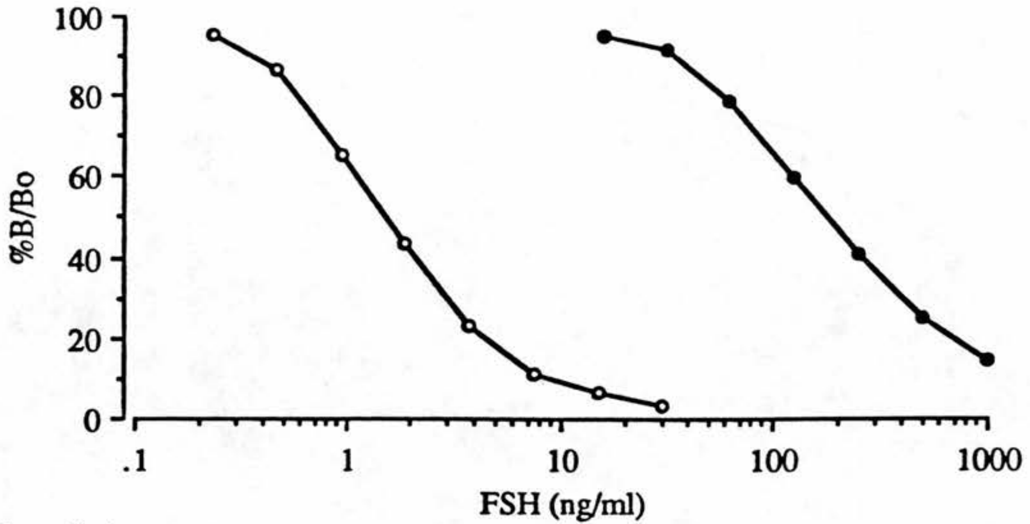

Fig. 2.4.
Comparison of the standard curves for the S9 FSH standard in the M/91 assay (•) and the RP1 standard in the NIAMMD - I - 1 assay (○). %B/Bo represents the label bound as a percentage of the binding in the presence of no unlabelled hormone: each point is the mean of triplicate determinations.

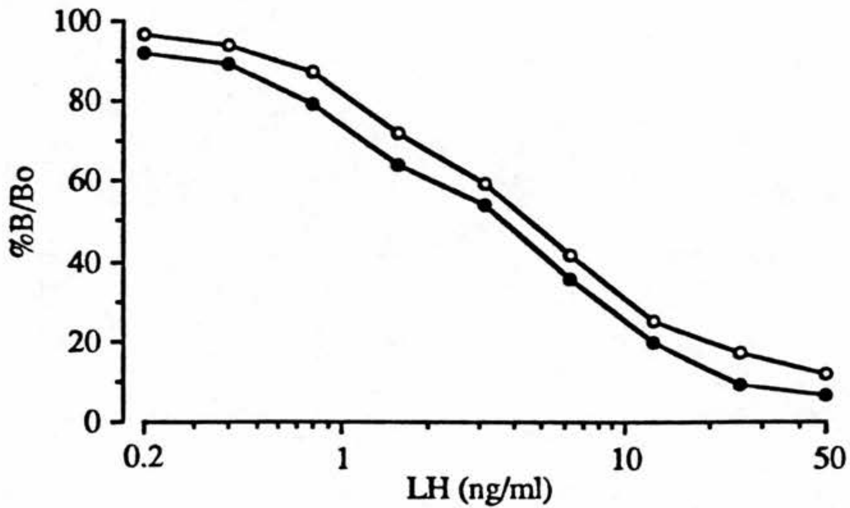

Fig. 2.5.
Comparison of the standard curves for the S18 (•) and the S25 (○) LH standards. %B/Bo represents the label bound as a percentage of the binding in the presence of no unlabelled hormone: each point is the mean of triplicate determinations.

In the later experiments the NIH - oLH - S18 standard was replaced by a new standard, NIH - oLH - S25. This standard was used in the same range as the S18 and gave a similar standard curve (Fig 2.5.). The sensitivity of this assay was in the range 0.2 - 0.3ng NIH - oLH - S25/ml and the intra and inter assay coefficients of variation 7.2% and 11.6%. The protocol for this assay can be found in the appendix (Section 10.1.5).

2.10. Steroid Hormones Assays

2.10.1. Progesterone Assay

Progesterone was measured in plasma by a direct non - extraction radioimmunoassay using a sheep anti progesterone antibody (361) at a final dilution of 1:35000, ^{125}I - labelled progesterone 11α glucuronide tyramine conjugate as tracer and pregn - 4 - ene - 3,20 dione (Sigma, Poole, Dorset, U.K.) as standard in the range 0.08 - 10 ng/ml. The assay was similar to that described by (Djahanbakhch et al., 1981), validated for the measurement of progesterone in extracted sheep plasma (McNeilly, 1984). The assay was modified to allow direct measurement in plasma by lowering the buffer pH to 6 and adding 8 - anilino - 1- naphthalene sulphonic acid (100μg/tube; Sigma) to displace binding of progesterone to carrier proteins (McNeilly & Fraser, 1987). A volume of plasma from an ovariectomised ewe equal to the volume of standard used was added to the NSB, B_O and standard tubes. Separation of bound and free tracer was performed by addition of 15% polyethlyene glycol and centrifugation at 2500 rpm for 30 min. The sensitivity of this assay was 0.1 ng/ml and the intra and inter assay coefficients of variation 8.4% and 13.2%. The protocol for this assay can be found in the appendix (Section 10.1.2).

2.10.2. Oestradiol Assay

Oestradiol was measured in plasma following extraction with diethly ether (Baird et al., 1981) using sheep anti - oestradiol antibody (BW 26/9/82) at a final dilution of 1:4000000, ^{125}I oestradiol - 17β as tracer, and 0estradiol - 17β as standard in the range 20 - 500 pg/ml. The sensitivity of this plasma assay was 25pg/ml and the intra and inter assay coefficients of variation 10% and 12%. The same assay was used to measure oestradiol in culture medium but samples were assayed directly without prior extraction.The sensitivity of this assay was 60 pg/ml and the intra and inter assay

coefficients of variation 10% and 14%. The protocol for both the extraction and non-extraction assays can be found in the appendix (Section 10.1.7).

2.10.3. Androstenedione Assay

Androstenedione was measured in plasma by a previously described radioimmunoassay (Thomson et al., 1989) modified for use in sheep plasma (Campbell et al., 1990). The assay used an antiserum raised in a rabbit against androstenedione - 3 - carboxymethyloxime - BSA at a final dilution of 1:112000 with androstenedione - 3 - carboxymethyloxime linked to ^{125}I - histamine as tracer and 4 - androstene - 3,17 dione as standard in the range 4 - 250 pg/ml. Prior to assay plasma samples were extracted using 10 volumes of a 4:1 hexane:ether mixture. The sensitivity of the assay was 40 pg/ml and the intra and inter assay coefficients of variation 7.1 and 13.2%. Androstenedione was measured in culture medium using the same assay but without prior extraction. The sensitivity of the assay for culture medium was 45 pg/ml and the intra and inter assay coefficients of variation 8.6 and 12.8%. The protocol for both the extraction and non-extraction assays can be found in the appendix (Section 10.1.8).

2.11. Plasma Hormone Binding Capacities

2.11.1. Inhibin Binding Capacity

Inhibin binding capacity was determined in the plasma of immunized ewes by measuring the binding of ^{125}I-labeled p1-26α inhibin peptide in plasma collected at various time intervals following immunization. Incubations were carried out in 0.075M phosphate buffered 0.9% saline (pH 7.4) containing 1% bovine serum albumin and 0.01% thiomersalate. The ^{125}I - labeled p1-26α inhibin peptide was made up to 15 000 cpm in the buffer and 0.1ml tracer, 0.1ml plasma dilution, and 0.1ml buffer (final volume 0.3ml) incubated in 10mm x 65mm plastic tubes overnight at 4°C. Bound tracer was then separated by adding 1ml of 15% PEG + 0.1% Triton-X in 0.9% saline and centrifuging at 2000g for 30 minutes. The precipitate (bound fraction) was then counted and binding expressed as a percentage of the total counts added.

2.11.2. Oestradiol Binding Capacity

Oestradiol binding in the plasma of immunized ewes was determined by measuring the binding of ^{125}I - labeled oestradiol by plasma samples at a final diluition

of 1:1200. Incubations were carried out in 0.075mol phosphate-buffered 0.9% (w/v) saline/l (pH 7.2) containing 1% (w/v) gelatin and 0.01% (w/v) thiomersalate (PGBS). The 125I - labelled oestradiol was made up to 15 000 cpm in the buffer and 0.1ml tracer, 0.1ml plasma dilution and 0.1ml buffer (final volume 0.3ml) incubated in 10 x 75mm glass tubes overnight at 4°C. Bound tracer was then separated by adding 0.5ml Dextran Charcoal (0.125% dextran, 1.25% charcoal in PGBS) and centrifuging at 2000 g for 15 min. The supernatant (bound fraction) was then counted and binding expressed as a percentage of the total counts added.

2.12. Assay Counting and Data Reduction

In Chapters 3, 4, and 5 assay tubes were counted using a 5400 Series CRYSTAL™ Multidetector Manual Gamma Counter (Packard Instrument Company, Downers Grove, Il) with well type sodium iodide crystal detectors and a detection efficiency for ^{125}I of 75%. The machine calculates the % B_o and the % NSB and then calculates the net counts per min (Counts per min - NSB counts per min) for standards, quality controls and samples. The machine then calculates a standard curve using a 4 - parameter logistic fit which straightens out hyperbolic curves to provide straight line curves out to the end point parameters (B_o and NSB). The machine then calculates the ED_{20}, ED_{50} and ED_{80} as well as quality control and samples hormone concentration, giving the coefficient of variation for each quality control or sample. Sample values are rejected if they show poor replication (> 3 S.D.) or are out of the standard range.

In chapters 6, 7, 8 and 9 assay tubes were counted using a 1261 Multigamma Counter (Wallac oy, Turka, Finland) with well type aluminium - covered sodium iodide crystals and a minimum efficiency for ^{125}I detection of 75%. The counts were collected in a digital data logger (Mutek Data Grabber) and transfered to an Apple Macintosh computer on which calculations were made using the Assayzap assay calculation programme (Elsevier, Biosoft, U.K.). Assayzap plots a standard curve using a 4 - parameter fit which uses the B_o and NSB values to estimate the upper and lower limits of the curve and then finds the best estimated fit through the standard points. The programme then adjusts this fit by weighting individual points according to how closely they agree with the estimated fit, effectively ignoring outlying points. The programme keeps a record of quality controls and provides a constantly updated measure of the inter - assay coefficient of variation, as well as calculating the intra - assay coefficient of variation for each of the quality controls.

2.13. Statistical Analysis

Statistical analysis of data was carried out using an Apple Macintosh computer. Student's t - tests were carried out using the Statworks programme (Data Metrics Inc.), correlation analysis was performed using the Statview 512+ programme (Brainpower Inc., Calabasas, CA) and analysis of variance was performed using the CLR Anova (Clear Lake Research) programme. Pulse analysis was performed using the Munro pulse analysis programme (Elsevier, Biosoft, U.K.).

Chapter 3

Source of Ovarian Inhibin Secretion During the Oestrous Cycle

3.1. Introduction

Inhibin is produced by the granulosa cells of ovarian follicles (Henderson & Franchimont, 1981; Tsonis et al., 1983; Bicsak et al., 1988), high concentrations being found in the follicular fluid of all species studied, including sheep (Tsonis et al., 1983). There is also evidence to indicate that the corpus luteum secretes inhibin in women (Davis et al., 1987; McLachlan et al., 1987; Tsonis et al., 1987) and in mature rats, where mRNA for inhibin has been demonstrated in luteal tissue (Davis et al., 1986). Although it has also been suggested that the sheep corpus luteum secretes inhibin, as measured by bioassay (Tsonis et al., 1988a; Tsonis et al., 1988b) efforts to demonstrate inhibin gene expression in luteal tissue have failed in sheep and cattle (Rodgers et al., 1989), as have attempts to show inhibin production from cultured bovine luteal tissue (Henderson & Franchimont, 1983). There is, therefore, some uncertainty as to whether the sheep corpus luteum secretes inhibin.

As well as uncertainty over the source of inhibin production in the sheep, reports are contradictory over the changes in inhibin concentration during the oestrous cycle. Peripheral inhibin levels have been reported as remaining unchanged following luteal regression (McNeilly et al., 1989), at a time when FSH levels are falling, while Tsonis et al., (1988a & b) have reported a fall in concentration of bioactive inhibin at this time. Inhibin levels have, however, also been reported to rise during the follicular phase (Findlay et al., 1990; Campbell et al., 1990a), though the rise reported by Campbell et al. was not consistent between animals.

In this study we have investigated the source of inhibin secretion in the ovary of the sheep by comparing ovarian secretion rates before and after enucleation of the corpus luteum, and by comparing secretion rates from ovaries with corpora lutea to contralateral ovaries without luteal tissue. We have also investigated inhibin secretion rates and peripheral concentrations at different stages of the oestrous cycle, in order to see whether changes occur as the ewe progresses from the luteal phase through the follicular phase of the cycle.

3.2. Materials and Methods

3.2.1. Synchronisation and Grouping

The following experimental procedures were carried out during the breeding season, in November and December 1987, on 24 mature Scottish Blackface ewes. Oestrous cycles were synchronised as described in Section 2.2. The animals were then allocated to four groups of six animals with similar mean group weights (64.3±0.1 kg). Animals in group A were operated on during the mid luteal phase of the oestrous cycle (Day 10). Groups B, C, and D were operated on during the follicular phase 26.8±1.4, 36.6±0.8, and 59.8±0.3h following an i.m. injection of cloprostenol (125μg) on day 10 of the oestrous cycle.

3.2.2. Experimental Procedure

Anaesthesia was induced by minimal quantities (200-300mg) of a mixture of two parts thiopentone (Intraval; RMB Animal Health Ltd, Dagenham. Essex, U.K.) and one part pentobarbitone sodium (Sagatal; May and Barker Ltd, Dagenham, Essex, U.K.) and maintained using halothane (1.5 - 2.5%). The jugular vein was cannulated using a plastic cannula and three - way tap. Mid ventral laparotomy was then performed and the reproductive tract exteriorized. Both ovarian veins were cannulated 1 - 2 cm from the ovary using a 21 - gauge needle, and 5000 units of heparin administered i.v. through the jugular cannula. The proximal end of the ovarian vein was then occluded and two or three serial timed 10 ml samples of ovarian venous blood collected by syringe on both sides of the tract. The corpora lutea were then enucleated by blunt dissection, and further timed samples of ovarian venous blood collected. Jugular venous samples (20ml) were taken before and after enucleation of the corpora lutea. Finally, the ovaries were removed and all follicles ≥3mm dissected out. In jugular venous plasma LH, FSH, immunoactive inhibin and progesterone were measured by radioimmunoassay and in ovarian venous plasma immunoactive inhibin, progesterone, and oestradiol were measured. Where sufficient plasma was available bioactive inhibin was also measured in ovarian venous plasma.

3.2.3. Analysis of Data

Hormone levels at different stages of the oestrous cycle were analysed by analysis of variance and a Duncan's Multiple Range test. Hormone secretion rates were

calculated from the concentration of hormone in the ovarian vein, the flow rate, and the haematocrit (Baird et al., 1981). Secretion rates before and after enucleation of the corpus luteum were analysed by means of a Student's paired t -test, with pre- and post-enucleation hormone ratios being analysed by a Student's paired t -test on square root transformed data. When comparing inhibin secretion rates from ovaries with and without luteal tissue, and ovaries with and without follicles > 3mm, a Student's paired t -test was also used. The bioactive/immunoactive inhibin ratios were analysed using a rankit normality test (Wardlaw, 1987) and found to deviate from normal distribution, and so a non-parametric Mann Witney U test was applied. The metabolic clearance rate (MCR) of inhibin was calculated using the formula:

$$MCR = \text{Ovarian Secretion Rate} / \text{Peripheral Concentration}$$

Calculations were made for all animals using the mean secretion rate before enucleation of the corpus luteum, and the mean peripheral concentration of inhibin at that time.

3.3. Results

Following cloprostenol-induced luteal regression there was a rise in the concentration of LH and a fall in the concentration of FSH (Fig. 3.1.). This fall in FSH approached significance ($p<0.06$) in the group 36h following cloprostenol - induced luteal regression (3.10 ± 0.34 compared with 4.25 ± 0.41 ng/ml), and in the group 60h after cloprostenol concentrations of FSH were significantly ($p<0.05$) elevated (8.88 ± 1.84 ng/ml). Two of the ewes in the group 60h after cloprostenol - induced luteal regression were sampled during the LH surge (concentration > 10ng/ml) suggesting that the elevated concentration of FSH in this group was due to the coincidental periovulatory FSH peak.

Peripheral concentrations of progesterone were significantly ($p < 0.001$) lower at 24-30, 36, and 60h following cloprostenol-induced luteal regression compared to the 0 h luteal phase group (Fig. 3.2.), this fall being indicative of luteolysis. During the experiment there were no significant differences in the concentration of inhibin in peripheral blood between groups (Fig.3.2.), although a slight decline was evident which approached statistical significance ($p < 0.06$) by 60h.

When hormone secretion rates from both ovaries were examined, progesterone was once again significantly ($p < 0.01$) lower in the three groups in which luteolysis was induced by cloprostenol than in the luteal phase group (Fig. 3.3.). Inhibin secretion rate was not significantly different between the four groups, ranging from 2.4-3.4 ng/min. The secretion rate of oestradiol at 24-30h after injection of cloprostenol

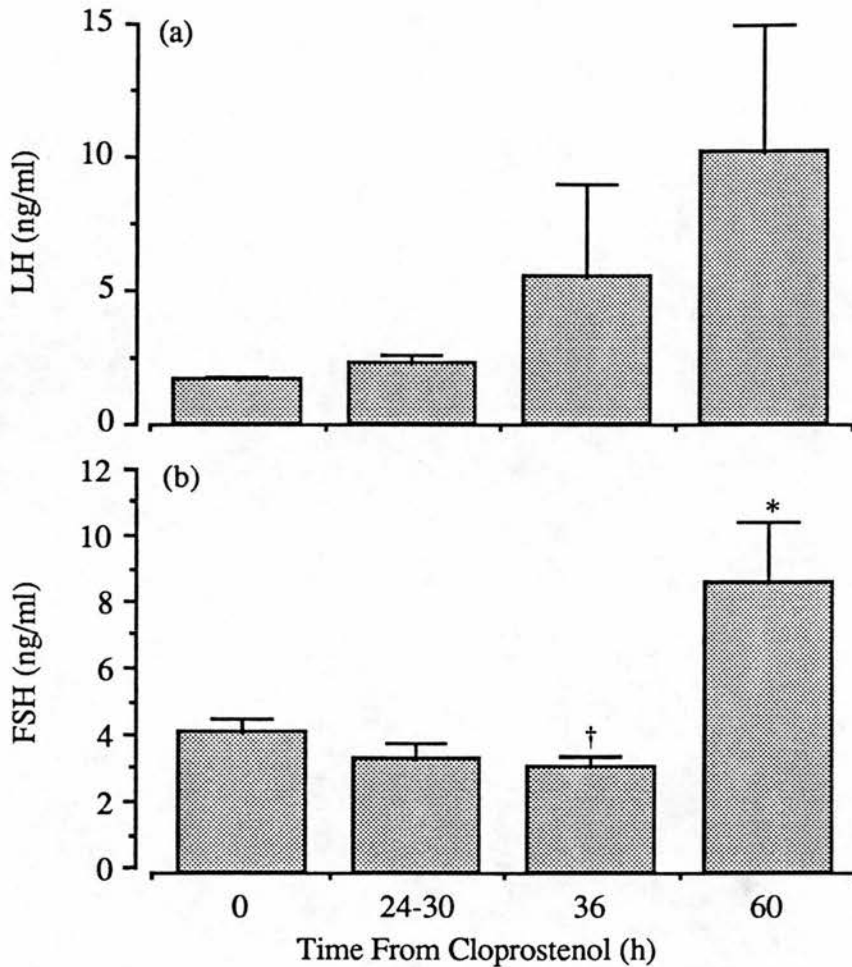

Fig. 3.1.
Mean (± S.E.M.) peripheral plasma concentrations of (a) LH and (b) FSH in sheep 0, 24-30, 36 and 60 h after i.m. injection with cloprostenol. ($n = 6$) † $p < 0.07$, * $p < 0.05$ compared to the 0 h group (analysis of variance and Duncan's multiple range test).

was significantly ($p < 0.05$) higher than during the luteal phase (2.37±0.61 compared with 0.78±0.20 ng/min), but by 60h after injection with cloprostenol the secretion rate of oestradiol had returned to a value similar to that found during the luteal phase (0.71±0.38 ng/min).

As expected, enucleation of the corpus luteum resulted in a significant ($p < 0.001$) fall in the secretion rate of progesterone (Fig. 3.4.). There was also a less marked fall in the secretion rate of both inhibin ($p < 0.001$) and oestradiol ($p < 0.01$). The changes in hormone secretion rate following corpus luteum enucleation are shown in a single representative animal in Fig. 3.5., which shows clearly the smaller fall in the secretion rates of inhibin and oestradiol. As the corpus luteum in the sheep does not produce oestradiol this result indicated that the fall in inhibin secretion rate might not

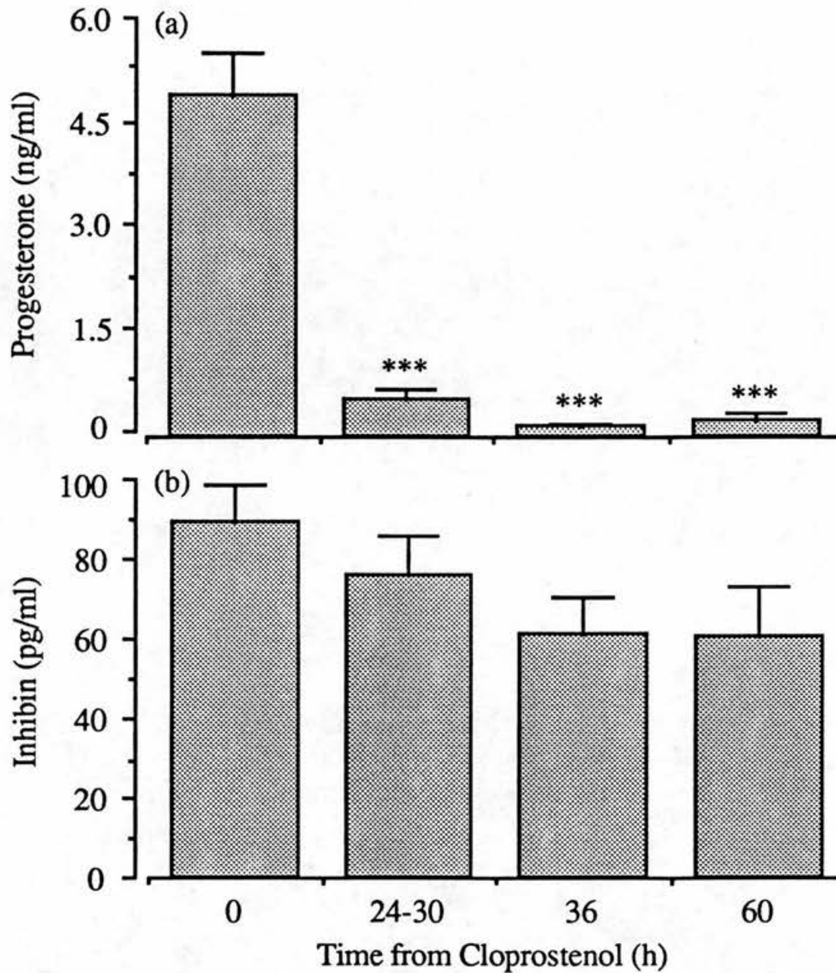

Fig 3.2.
Mean (± S.E.M.) peripheral plasma concentrations of (a) progesterone and (b) inhibin in sheep 0, 24-30, 36 and 60 h after i.m. injection of cloprostenol. (n = 6) *** $p < 0.001$ compared with 0 h group (analysis of variance and Duncans multiple Range test).

have been the result of the removal of secretion of inhibin by the corpus luteum. To clarify this problem progesterone to inhibin and oestradiol to inhibin ratios were compared before and after enucleation. While the progesterone to inhibin ratio fell significantly ($p < 0.001$) following enucleation (252.8±69.7 compared with 45.7±12.7), the oestradiol to inhibin ratio remained unchanged (0.61±0.15 compared with 0.57±0.14).

In 11 animals which contained luteal tissue in only one of the two ovaries, the secretion rate of progesterone, as expected, was significantly ($p < 0.05$) lower from the ovary without luteal tissue when compared with the contralateral one with a corpus luteum (Fig. 3.6.) (17.0 ± 5.2 ng/min compared with 322.7 ± 145.5 ng/min). The

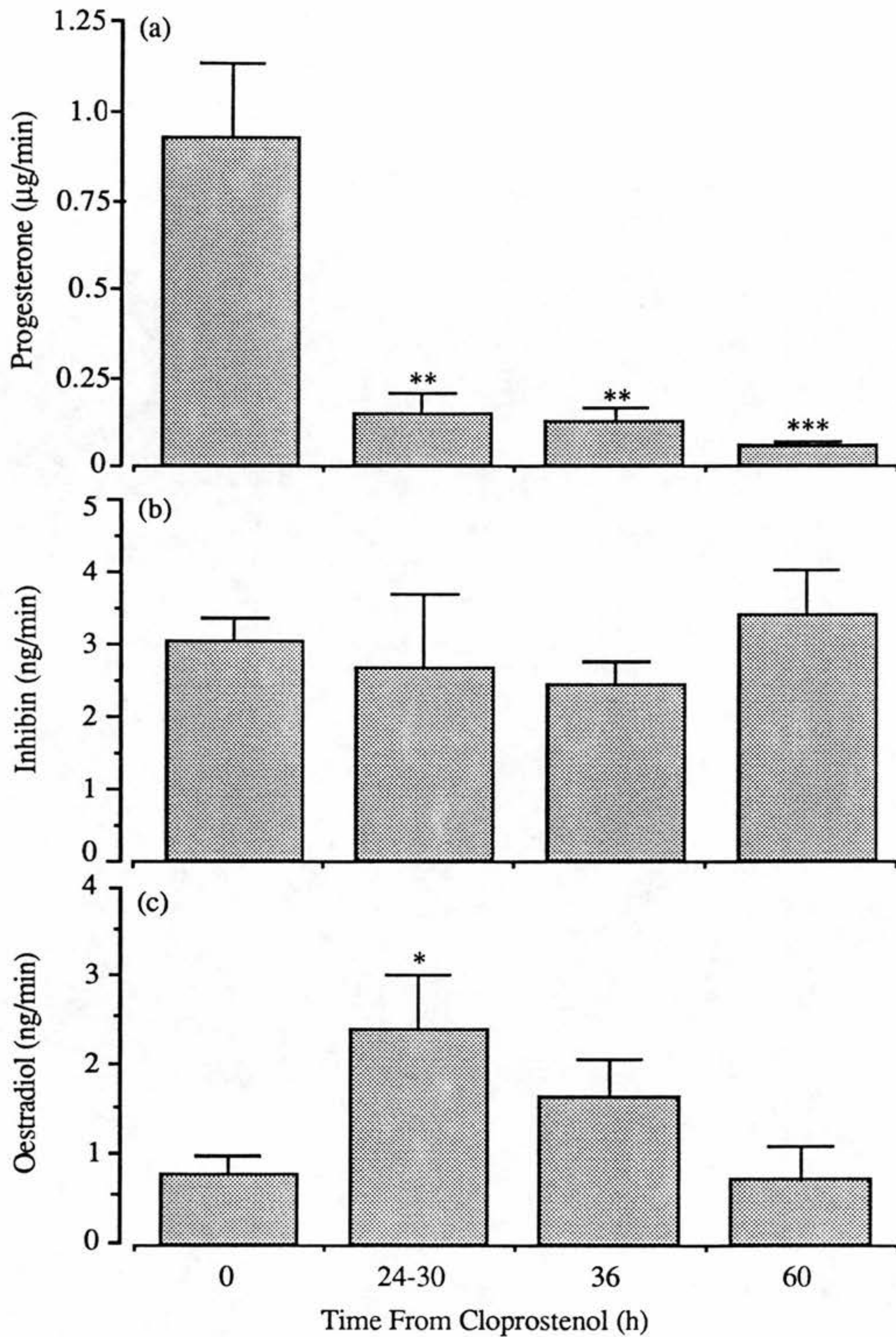

Fig. 3.3.
Mean (±S.E.M.) combined rate of ovarian secretion of (a) progesterone (b) inhibin and (c) oestradiol in sheep 0, 24-30. 36 and 60 h following i.m. injection with cloprostenol. (n = 6) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the 0 h group (analysis of variance and Duncan's Multiple Range test)

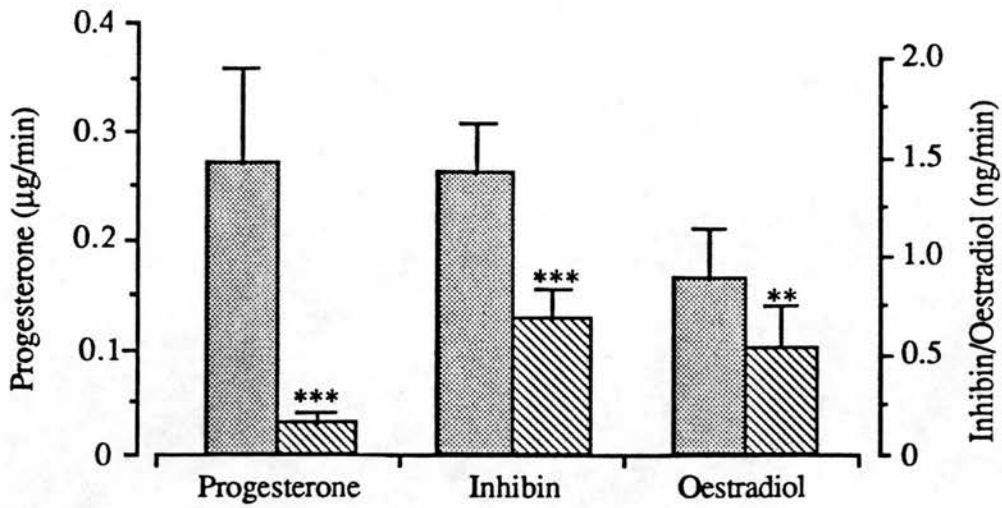

Fig. 3.4

Mean (± S.E.M.) rates of ovarian secretion of progesterone, inhibin and oestradiol before (▩) and after (▧) enucleation of the corpus luteum.** $p < 0.01$ *** $p < 0.001$ compared with the secretion rate before enucleation (Student's paired t - test).

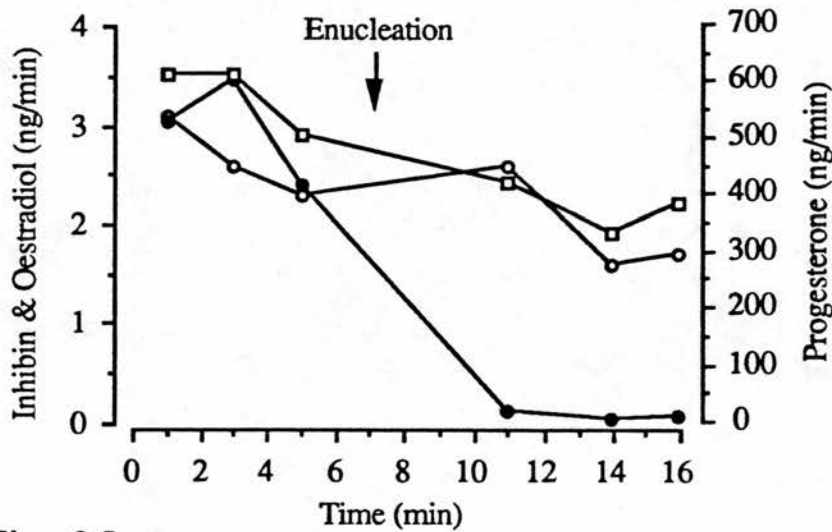

Fig. 3.5

The effect of corpus luteum enucleation on the ovarian secretion rate of inhibin(○), oestradiol (□) & progesterone (●) in a single representative animal.

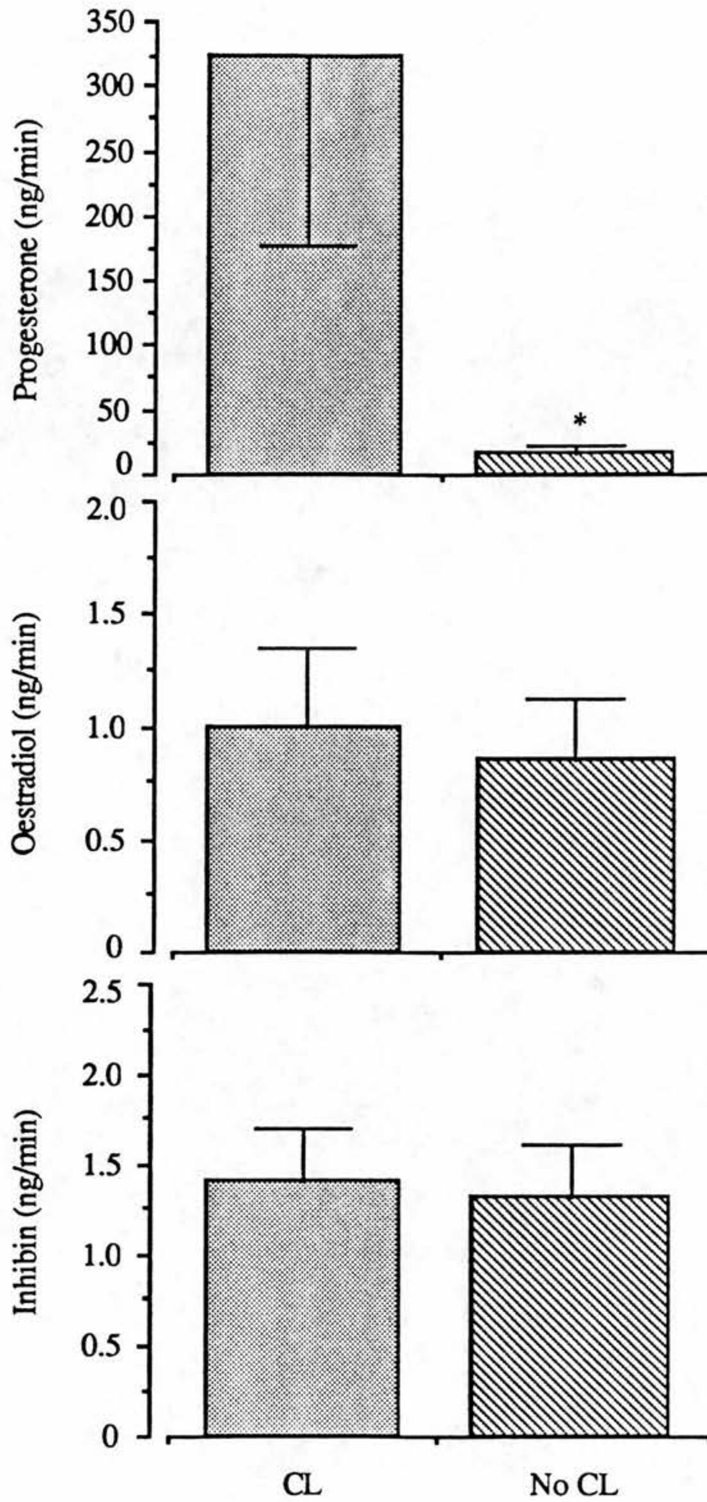

Fig. 3.6.
Mean (± S.E.M) ovarian secretion rates of progesterone, oestradiol and inhibin from ovaries with (▒) and without (▧) a corpus luteum (CL) (n = 11). * $p < 0.05$ (Student's paired t - Test).

secretion rates of inhibin and oestradiol, however, did not differ between ovaries with and without luteal tissue (inhibin, 1.41 ± 0.30 compared with 1.32 ± 0.30 ng/min; oestradiol, 1.00 ± 0.35 compared with 0.86 ± 0.27 ng/min). In four animals, one of the two ovaries contained no follicles ≥ 3mm while the contralateral ovary possessed a number of follicles above this size. As expected, in these animals the ovaries with larger follicles secreted significantly ($p < 0.05$) more oestradiol than the ovaries without follicles ≥ 3mm (1.75 ± 0.59 compared with 0.05 ± 0.01 ng/min) (Fig. 3.7.). Inhibin secretion was also significantly ($p < 0.01$) higher from ovaries with follicles > 3 mm than from the contralateral ovaries containing only small follicles ≤ 3 mm (2.28 ± 0.36 compared with 0.25 ± 0.06 ng/min).

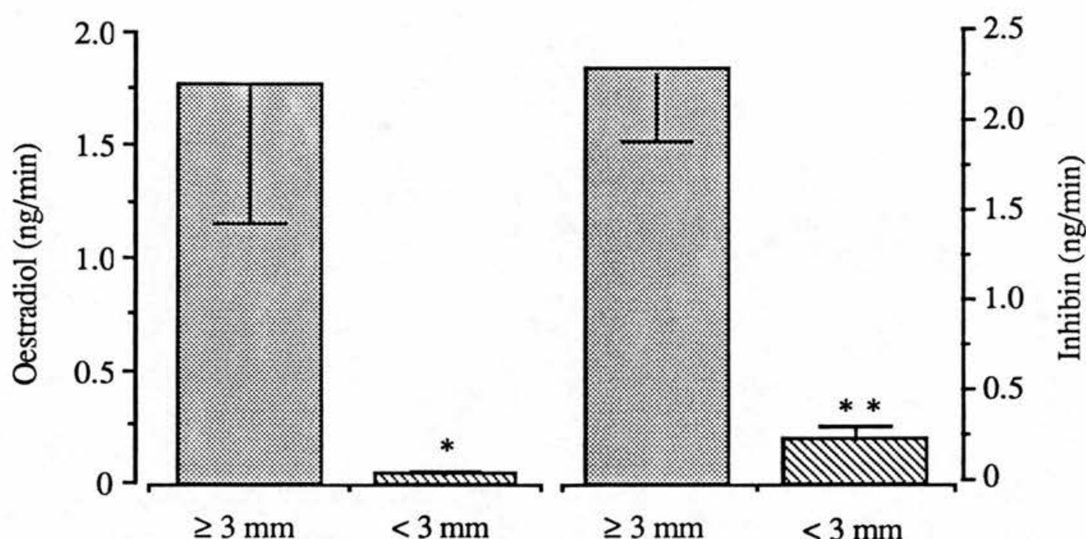

Fig. 3.7.
Mean (± S.E.M) ovarian secretion rates of oestradiol and inhibin from ovaries with (▩) and without (▧) follicles ≥ 3 mm (n = 4). * $p < 0.05$, ** $p < 0.01$ (Student's paired t - Test).

In animals in which the concentrations of inhibin in the ovarian vein were sufficiently high and enough plasma was available, the concentration of bioactive inhibin was measured. The bioactive and immunoactive concentrations of inhibin in these samples showing good correlation ($y = 0.49+0.54x$, $r = 0.71$; $p < 0.001$) (Fig. 3.8.). As a result of this high correlation the pattern of bioactive inhibin secretion between the four groups was similar to that of immunoactive inhibin (Fig. 3.9.), showing no significant differences between groups. The ratio (U:ng) of bioactive to

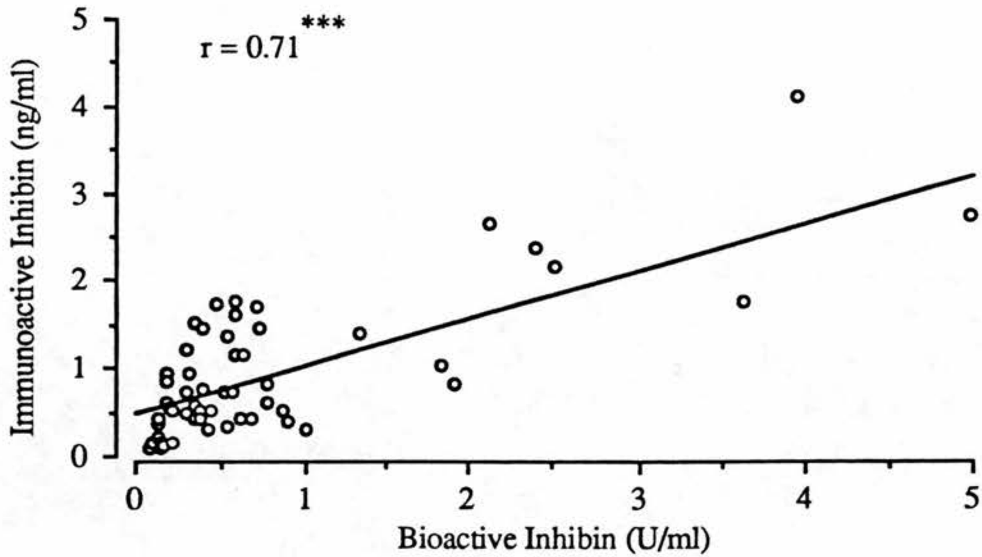

Fig. 3.8.
Correlation between immunoactive & bioactive inhibin concentrations in ovarian venous plasma samples on which a bioassay was performed. (n = 57) *** $p < 0.001$ (Regression analysis).

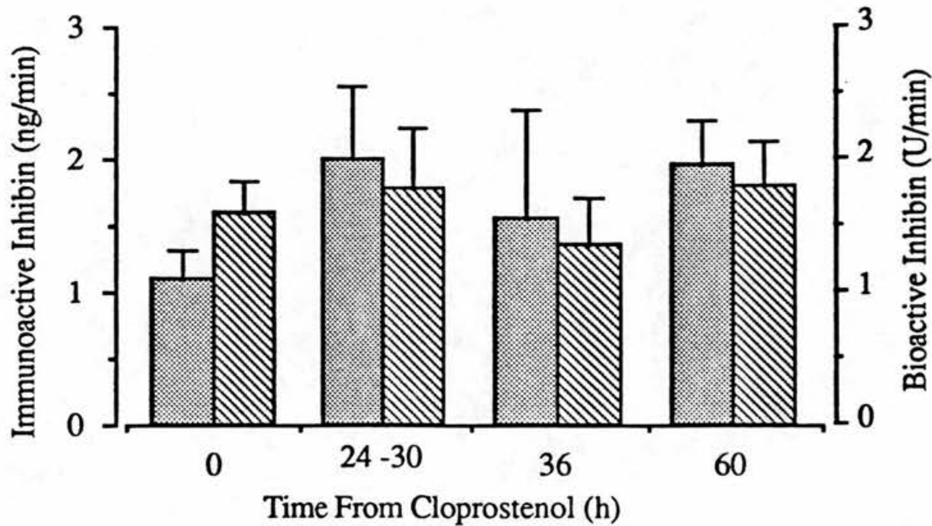

Fig. 3.9.
Mean (± S.E.M.) rates of secretion of immunoactive (▩) and bioactive (▧) inhibin per ovary in sheep 0 (n = 12), 24 - 30 (n = 10), 36 (n = 6) and 60 (n = 11) h after i.m. injection with cloprostenol, based on samples on which inhibin bioassays were performed.

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immunoactive inhibin was 0.68 (range 0.30 - 1.40) in the luteal phase (n=17), and 1.04 (range 0.18 - 3.37) in the follicular phase (n=40). While the ratio was higher during the follicular phase, this difference was not statistically significant.

The MCR of inhibin was 43.0±4.9 ml plasma/min (n=24).

3.4. Discussion

The hormone secretion rates in this study were based on samples collected by *in situ* cannulation of the ovarian veins in anaesthetized ewes. Despite the fact that the ewes were under anaesthetic, the secretion rates were similar to those found in a previous study on ewes in which the left ovary and its vascular pedicle had been autotransplanted to the neck, to allow the secretion rate of ovarian hormones to be measured in the conscious animal (McNeilly et al., 1989). The similarity in the pattern of progesterone secretion by the ovaries and the concentration in jugular venous plasma following luteal regression, also suggests that the *in situ* cannulation accurately reflects the true ovarian secretion *in vivo*.

In this study we found no significant changes in the concentration or secretion rate of immunoactive or bioactive inhibin following luteal regression, a finding similar to that of McNeilly et al. (1989). Other studies have shown a fall in bioactive inhibin (Tsonis et al., 1988a & b), or a rise in immunoactive inhibin (Findlay et al., 1990; Campbell et al., 1990a) at this time, though the rise reported by Campbell et al. was not consistent between animals. One explanation for the different findings in immunoactive inhibin levels could lie in the assays used as our assay involves an antiserum raised to a fragment of the α-chain (McNeilly et al., 1989), while the assay used by Findlay et al. utilizes an antiserum raised to the whole inhibin molecule. However, both assays been shown to give similar results when used to measure ovarian vein plasma inhibin concentrations in the same samples (Campbell et al., 1990a). Breed differences in inhibin concentrations appear to exist and these could represent one possible explanation for the differing results. Inhibin secretion also shows marked episodic fluctuations (Campbell et al., 1989; McNeilly & Baird, 1989) and in cross-sectional studies the timing of collection of samples in relation to these fluctuations could influence the findings.

The correlation between immuno and bioactive inhibin in those samples assayed for both types of activity was highly significant. The immunoassay involves the use of an antiserum raised to the first 1 to 26 amino acids of the N-terminus of the α-chain of the inhibin molecule which raises the possibility that as well as measuring whole

inhibin molecules, this assay could be detecting free α-chains, which would cross-react completely with the antiserum. There is evidence for the existence of large quantities of free inhibin α-chain in cultured rat granulosa cells (Bicsak et al., 1988), and in bovine, but not ovine, follicular fluid (Knight et al., 1989). The fact that our α-chain assay and the whole molecule assay of Findlay et al. (1990) showed similar results when measuring different molecular weight forms of inhibin also indicates that free α-chains do not pose a problem in our ovine assay system. In the sheep, using immunoblotting, McNeilly et al. (1989) found no evidence for the existence of free α-chains, and found a good correlation between immunoactive and bioactive concentrations of inhibin in culture media from ovine follicles. These reports, together with the fact that in this study the immunoassay showed good correlation with the inhibin bioassay, which would not measure the non-bioactive free α-chains, indicates that the immunoassay is giving a true measure of whole inhibin molecule levels.

Inhibin has been reported to be secreted in forms with reduced bioactivity during the follicular phase of the human menstrual cycle (Robertson et al., 1988). In this study the ratio of bioactive to immunoactive inhibin was found to be higher during the follicular phase, though this difference was not statistically significant. It would appear in the sheep, therefore, that there is no such fall in the bioactivity of inhibin following luteal regression.

Inhibin and oestradiol are thought to act in combination to cause suppression of pituitary production of FSH (Martin et al., 1988). In this study the rate of secretion of inhibin and jugular concentration, as well as peripheral FSH levels, were slightly lower in the two groups 24-30 and 36h after cloprostenol-induced luteal regression than in the mid luteal group. These results suggest that changes in inhibin levels are not responsible for the decline in FSH levels seen after luteal regression, and that oestradiol, the rate of secretion of which was significantly higher in the two groups 24-30 and 36h after injection with cloprostenol than in the luteal phase group, is the major modulator of this change. The low MCR for inhibin (43 ml/min) found in this study is of a similar order of magnitude to that found in a previous study (21.3±1.2ml/min; McNeilly et al., 1989) when compared to a much higher value reported for oestradiol of 1600 ml/min (Challis et al., 1970). This indicates that inhibin may be involved in the more long term regulation of FSH levels, while oestradiol with its much faster clearance rate may act as the regulator of more acute changes, as well as interacting with inhibin in longer term regulation. The hypothesis that oestradiol is more important than inhibin in regulating the fall in FSH seen at this time is supported by the fact that in the 60h group, where FSH levels were increased, the rate of secretion of oestradiol

showed a significant fall below that seen earlier in the follicular phase, while the rate of secretion of inhibin showed no such fall.

During the experiment, hormone secretion rates showed a slight tendency to decline, probably as a result of the anaesthesia. This was accentuated by the disruption to blood flow caused by the process of enucleation of the corpus luteum. As a result of this, following enucleation a significant decline was observed in the secretion rate of oestradiol, which is produced entirely by the follicles in the sheep (Baird & Scaramuzzi, 1976). A fall was also observed in inhibin secretion rate following enucleation, this fall appearing to be more closely related to the decline in oestradiol than to the large fall seen in progesterone secretion. To investigate whether the fall in secretion of inhibin was the result of the removal of a secretory source (the corpus luteum) like the fall in progesterone, or an artifact like the fall in oestradiol, the ratios of progesterone to inhibin and oestradiol to inhibin were compared before and after enucleation. The results showed that inhibin was following the same pattern as oestradiol, suggesting that inhibin is being produced largely, if not entirely, by the follicles, and not by the corpus luteum. This hypothesis was strengthened when hormone secretion rates were compared from ovaries with and without luteal tissue in the same animal. While significantly more progesterone was produced by ovaries with luteal tissue, no differences were found in the secretion rate of inhibin or oestradiol between the two ovaries. It would seem unlikely, therefore, from this data that the ovine corpus luteum secretes any significant amount of inhibin, despite previous reports to the contrary (Tsonis et al., 1988a & b). When rates of secretion of inhibin were compared from ovaries with and without large antral follicles (≥3mm) in the same animal, secretion of inhibin was significantly higher from ovaries with large antral follicles, ovaries without large antral follicles secreting low levels of inhibin in all cases. This finding was similar for oestradiol, suggesting that it is the large antral follicles which are the most important source of inhibin production. This idea is supported by the findings of Rodgers et al. (1989) who demonstrated the presence of α and β_A-inhibin mRNA in the antral follicles of sheep and cattle, but were unable to show mRNA for inhibin in either cyclic or pregnancy corpora lutea in sheep, and could only show low, declining, levels in early bovine corpora lutea.

In a previous study, Tsonis et al. (1988a) reported that the concentration of bioactive inhibin was higher in blood draining ovaries with luteal tissue than ovaries without, and that ovaries with multiple corpora lutea secreted more inhibin than those with a single corpus luteum. It should, however, be noted that in the earlier study Merino ewes, with a lower ovulation rate than Blackface ewes, were used, and that utero-ovarian rather than ovarian venous blood was collected, the concentrations of

ovarian hormones obviously differing between the two blood types. The earlier study also involved the pooling of results from normal ewes with ewes immunized against androstenedione which have been shown to secrete higher levels of inhibin due to the presence of larger numbers of follicles (Campbell et al., 1988), this larger number of follicles being reflected in a higher ovulation rate and hence a higher number of corpora lutea. It would therefore seem likely that in the earlier study more of the ovaries with a corpus luteum came from immunized animals, and as the Merino ewe usually has a single ovulation, most of the ovaries with two corpora lutea probably originated from immunized ewes. This would mean that a disproportionate number of the ovaries with luteal tissue were coming from immunized animals, with larger numbers of inhibin-secreting antral follicles, and would explain why ovaries with luteal tissue appeared to be secreting more inhibin.

From the results of this study we conclude that, in the sheep, the corpus luteum secretes very little, if any bioactive or immunoactive inhibin, and that large antral follicles are by far the main source of production of ovarian inhibin. We also conclude that inhibin does not appear to be responsible for the fall in FSH seen during the follicular phase of the oestrous cycle in the ewe, oestradiol being more important in this aspect of FSH regulation.

Chapter 4

Hormone Production from Follicles at Different Stages of the Oestrous Cycle

4.1. Introduction

The results of Chapter 3 indicated that in the sheep, the corpus luteum does not secrete a significant amount of inhibin into the ovarian vein, and that antral follicles greater than 3 mm in diameter are by far the main source of ovarian inhibin. Attempts to show inhibin gene expression in ovine luteal tissue have failed (Rodgers et al., 1989), supporting the suggestion that the ovine corpus luteum does not produce inhibin, while in the same study Rodgers et al. were able to show inhibin gene expression in ovine follicles. A number of studies have demonstrated inhibin production from the granulosa cells of ovarian follicles (Henderson & Franchimont, 1981; Tsonis, 1984; Bicsak et al., 1988) and it seems reasonable to assume that follicles are the main source of ovarian inhibin in the sheep. However, little is known about the inhibin production from different individual follicles, for example oestrogenic and atretic follicles, at different stages of the cycle.

Large antral follicles are also known to be the main source of ovarian oestradiol production (Baird & Scaramuzzi, 1976) and oestradiol secretion is known to increase as ewes progress from the luteal phase through the follicular phase (Baird & McNeilly, 1981) due to the emergence of highly oestrogenic follicles. Changes in inhibin concentration at this time are, however, less clear. In Chapter 3 a small decline in both inhibin secretion rate and peripheral concentration was observed at this time while other studies have reported no change in inhibin secretion (McNeilly et al., 1989) or a rise in inhibin secretion (Findlay et al., 1990; Campbell et al., 1990a). As the rise in oestradiol secretion at this time can be explained in terms of the development of highly oestrogenic follicles destined for ovulation, it may be that a detailed study of inhibin production from individual follicles may be able to add further to an understanding of the changes in inhibin secretion at this time.

In the ewe follicle selection is thought to occur during the early follicular phase, from the pool of small antral follicles present at that time (McNatty, 1982; Tsonis et al., 1984a; Webb et al., 1989), the selected oestrogenic follicle(s) emerging by 10h following luteolysis (McNatty et al., 1982). The decline in FSH concentration at this time is thought to be important in controlling the number of follicles that develop to ovulation (Baird, 1983) and the increase in oestradiol secretion from "selected"

follicle(s) is thought to be the main factor regulating this decline in FSH, though inhibin may also be important. By investigating the hormonal characteristics of individual follicles it may be possible to further clarify the role of inhibin during this important period.

In this study we investigated the hormonal characteristics of individual follicles in ewes during the luteal phase and at 3 stages of the follicular phase, as well as patterns of follicle development and hormonal characteristics.

4.2. Materials and Methods

This experiment was performed using the ovaries collected from the animals in the *in situ* cannulation experiment described in Chapter 3 and so methods will only be described in detail where differences from the previous experiment exist.

4.2.1. Synchronisation and Grouping

The following experimental procedures were carried out during the breeding season, in November and December 1987, on 24 mature Scottish Blackface ewes. Oestrous cycles were synchronised and animals were then allocated to four groups of six animals with similar mean group weights (64.3±0.1 kg) and operated on during the mid luteal phase of the oestrous cycle (Day 10) or during the follicular phase 26.8±1.4, 36.6±0.8, or 59.8±0.3h following a further injection of cloprostenol (125μg) on day 10 of the luteal phase.

4.2.2. Experimental Procedure

Anaesthesia was induced and mid ventral laparotomy was then performed and the reproductive tract exteriorized. Both ovarian veins were cannulated and serial timed samples of ovarian venous blood collected by syringe on both sides of the tract. The ovaries were removed to allow follicle dissection. Follicles judged to be greater than 3mm in diameter were first removed from the ovary by rough dissection, and then the surrounding stroma was removed under a stereoscopic microscope. Each follicle was then placed in an individual well of a culture plate and incubated for 2h in 1ml of Medium 199 (Flow Laboratories) at 37°C. Follicles were then removed from the culture medium and frozen. Later the antral fluid of each follicle was made up to 1ml in

phosphate buffered 0.9% saline for hormone measurement. To do this the volume of each follicle was estimated from its diameter according to the formula:

$$\text{Volume} = 2 \times (\text{Diameter}^{2.7}) \quad \text{(Carson et al., 1981)}$$

4.2.3. Analysis of Data

Hormone secretion rates were calculated from the concentration of hormone in the ovarian vein, the flow rate, and the haematocrit (Baird et al., 1981) and hormone levels at different stages of the oestrous cycle analysed using analysis of variance and Duncan's Multiple Range test. The production of hormone during incubation was correlated to follicular diameter and to the secretion rate into the ovarian vein *in vivo* by regression analysis. The number of each type of follicle present at different stages of the oestrous cycle was analysed by analysis of variance on data that had been transformed ($\sqrt{(x + {}^1/_2)}$ due to the presence of no follicles of a particular class in some animals in certain groups, while hormone concentrations in follicular fluid and incubate medium were compared between different follicle types by analysis of variance on untransformed data. A follicle was classified as being oestrogenic if the follicular fluid oestradiol concentration was > 200 ng/ml and the production of oestradiol during the 2h incubation period was > 25 pg/min. Follicles were then classified as small or large based on a size of < 4mm or ≥ 4mm. The small follicles (< 4mm) were all non - oestrogenic while the large follicles were sub divided into oestrogenic and non - oestrogenic groups.

4.3. Results

4.3.1. *In Vivo* Results

The peripheral hormone concentrations and hormone secretion rates into the ovarian veins have been discussed in detail in Chapter 3 and will only be briefly described here in order to allow the hormonal conditions at the time of ovary removal to be established. Following cloprostenol - induced luteal regression at time 0h there was a rise in the concentration of LH, and a fall in the concentration of FSH ($p < 0.06$) (Fig. 4.1 (a) and (b)). In the group operated on 60h following luteal regression LH concentration was > 10ng/ml in 2 of the animals indicating the occurrence of an LH surge, and suggesting that the elevated concentration of FSH seen in this group ($p < 0.05$) was due to the coincidental periovulatory FSH peak. The secretion rate of

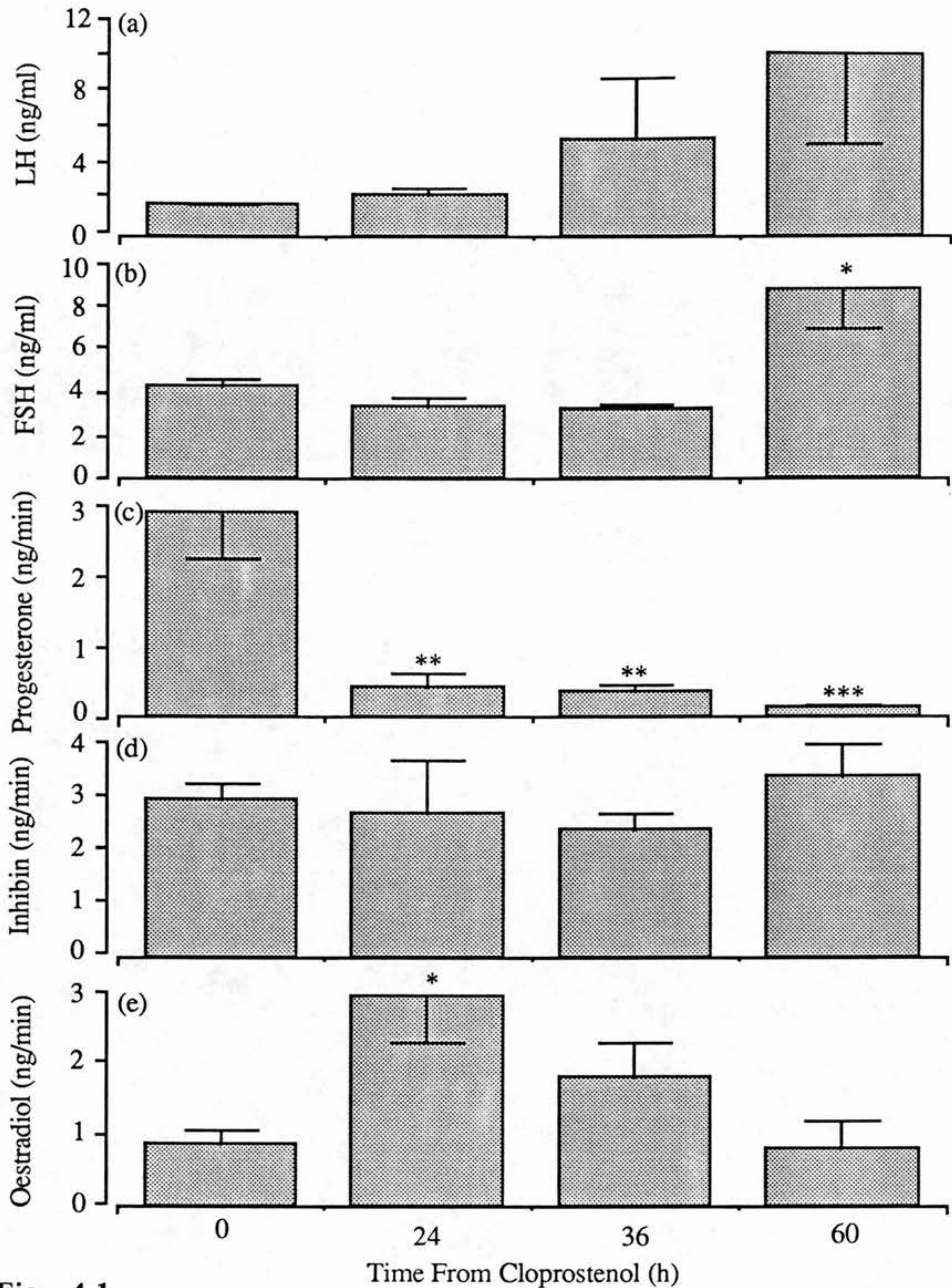

Fig. 4.1.
Mean (± S.E.M.) peripheral plasma concentrations of a) LH and b) FSH and mean (±S.E.M.) combined secretion rates into both ovarian veins of c) progesterone, d) inhibin and e) oestradiol in groups of 6 ewes operated on at day 10 of the luteal phase (time = 0) or during the follicular phase 24 - 30, 36, or 60h following an injection of 125µg cloprostenol on day 10 of the luteal phase to induce luteal regression. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the luteal phase group, analysis of variance followed by Duncan's Multiple Range test).

progesterone was significantly ($p < 0.01$) lower in the 3 groups following cloprostenol injection indicating that luteal regression had occurred (Fig 4.1 (c)) while the secretion rate of oestradiol was significantly ($p < 0.05$) elevated in the group 24 - 30h following luteal regression but returned to luteal phase levels by 60h (Fig. 4.1 (e)), while inhibin secretion rate did not differ significantly between the four groups (Fig. 4.1 (d)).

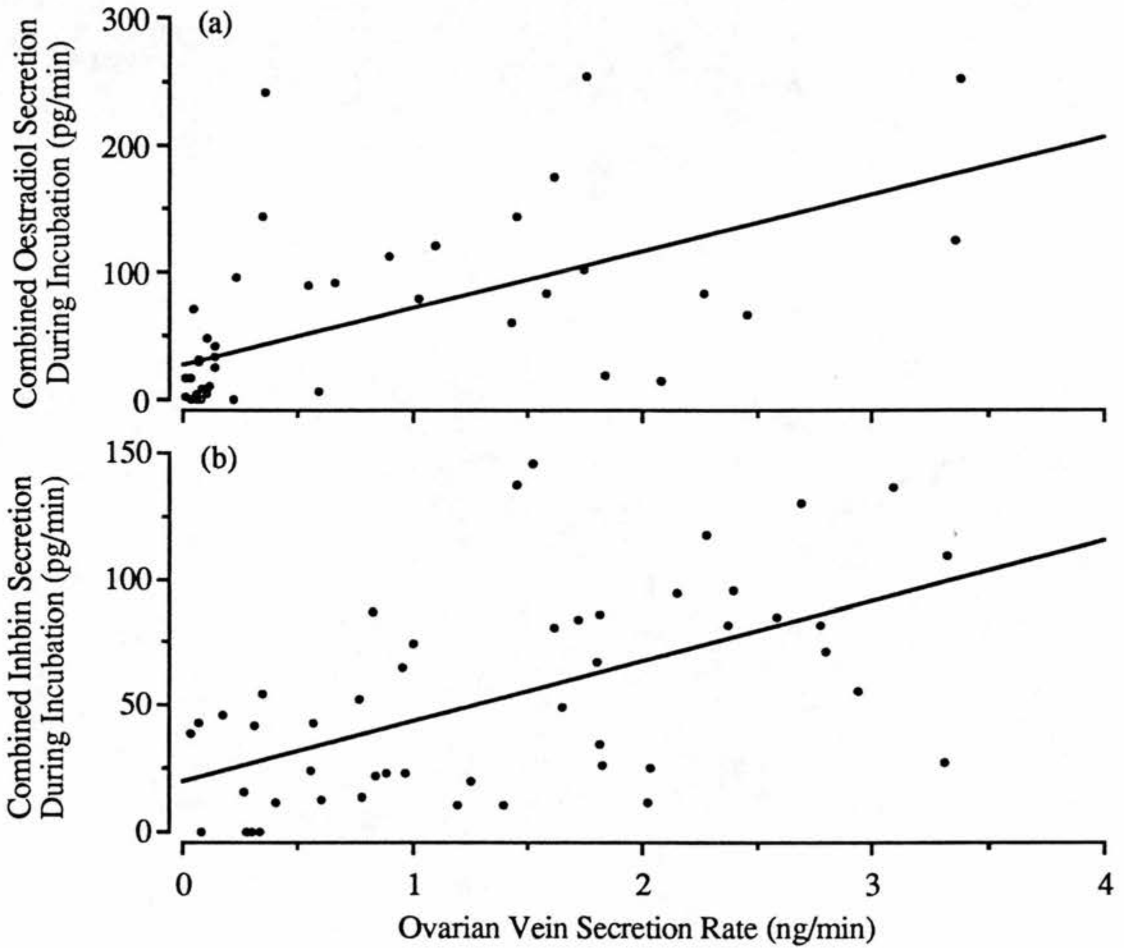

Fig. 4.2.
Correlation between the hormone secretion rates into the vein draining each ovary and the combined *in vitro* hormone secretion from all the follicles dissected from that ovary for (a) oestradiol ($n = 48$; $R = 0.60$; $p < 0.001$) and (b) inhibin ($n = 48$; $R = 0.57$; $p < 0.001$, regression analysis).

4.3.2. *In Vitro* Results

Good correlation was found between the concentration in follicular fluid and hormone production during incubation for oestradiol (correlation coefficient, R = 0.92) inhibin (R = 0.72) and androstenedione (R = 0.51) (all $p < 0.001$). There were, however, large differences in the ratio of follicular fluid concentration and hormone production during incubation between hormones. During the incubation period the total amount of androstenedione secreted represented 6.7 ± 0.4% of that remaining in the follicular fluid. This proportion of androstenedione was significantly ($p < 0.001$) greater than that of oestradiol (2.5 ± 0.2) or inhibin (0.5 ± 0.0.4). The production of oestradiol was, in turn, significantly ($p < 0.001$) greater than that of inhibin. There was also good correlation when the ovarian secretion rates of both inhibin and oestradiol into each ovarian vein *in vivo* were compared to the total combined rates of hormone production during culture from all the follicles dissected from that ovary (oestradiol - R = 0.60; inhibin, R = 0.57; both $p < 0.001$) (Fig 4.2.). The *in vivo* secretion rate was, however, 20 - 30 times larger than the rate of *in vitro* hormone production.
When inhibin production from follicles during incubation was compared to their diameter good correlation was found (R = 0.72; $p < 0.001$), while a similar comparison for oestradiol resulted in a somewhat lower correlation coefficient (R = 0.56; $p < 0.001$) (Fig 4.3.). While 90% of follicles greater than 4 mm in diameter secreted at least 10 pg/min of inhibin into the culture medium over 60% of follicles above this size secreted less than 25 pg/min, and a number of follicles as large as 6 - 9 mm secreted only minimal quantities of oestradiol. Androstenedione production during incubation was also correlated with follicle diameter, though less strongly than oestradiol or inhibin (R = 0.39; $p < 0.01$). Oestradiol and inhibin concentrations in the follicular fluid were also correlated with follicles diameter (oestradiol, R = 0.55; inhibin, R = 0.49; both $p < 0.001$) while androstenedione concentration in the follicular fluid did not correlated significantly with follicle diameter (R = 0.09).

When the *in vitro* production of oestradiol in relation to follicle size was compared in the four groups of ewes individually all the groups were found to contain a number of follicles of various sizes up to 7 - 9mm which secreted minimal quantities of oestradiol (Fig 4.4.). However, there were large differences in the number of follicles secreting significant quantities of oestradiol. In the luteal phase group there were 4 follicles that secreted > 25pg oestradiol/min compared to 7 follicles in the group operated on 24 - 30h following luteal regression and 12 such follicles in the 36h group, while in the 60h group there were only 3 such follicles. The number of these follicles in the 36h group was significantly greater than the number in the luteal phase and 60h

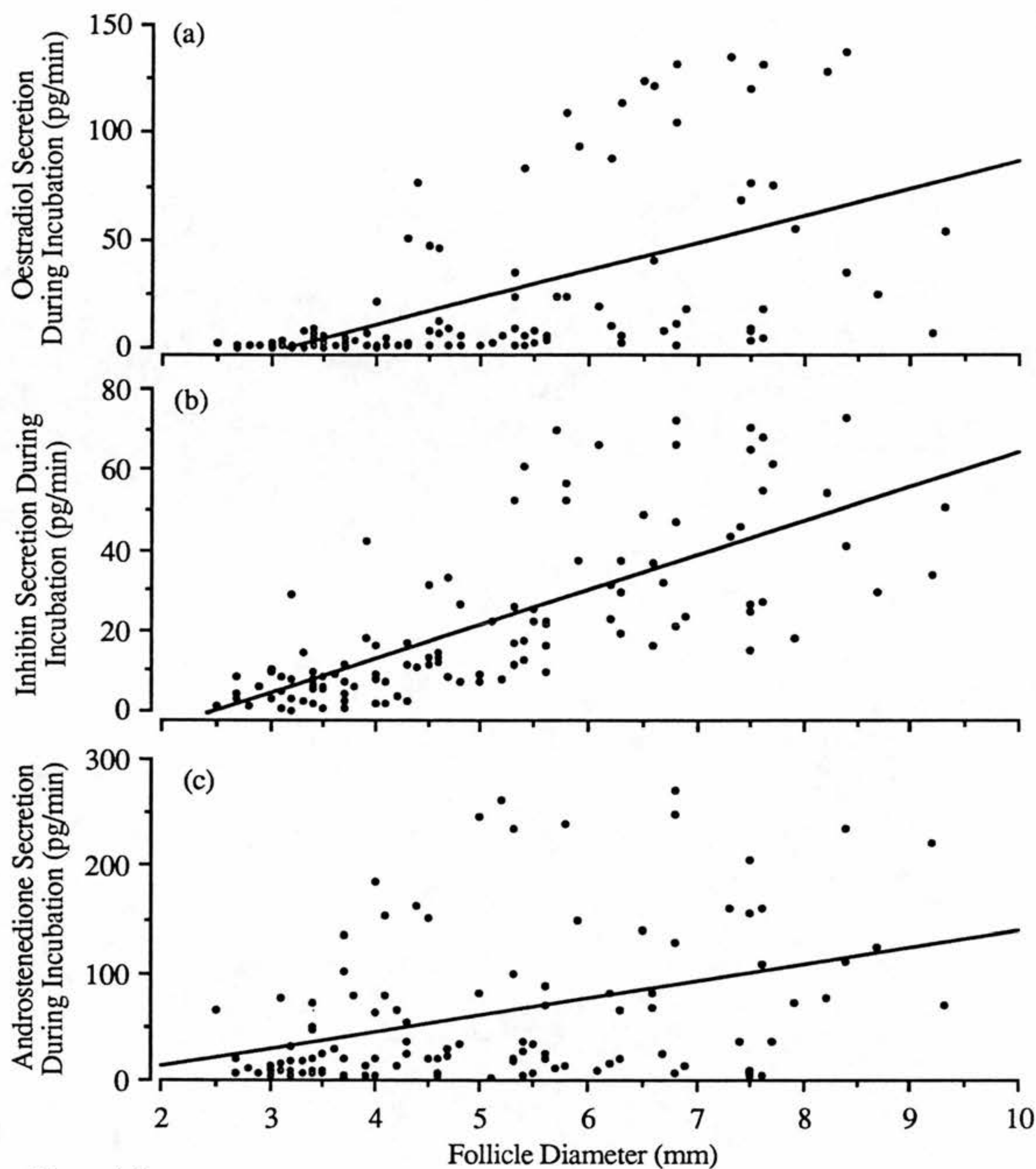

Fig. 4.3.

Correlation between follicle diameter and the rate of hormone secretion *in vitro* for a) oestradiol ($n = 113$; $R = 0.56$; $p < 0.001$) b) inhibin ($n = 113$; $R = 0.72$; $p < 0.001$) and c) androstenedione ($n = 111$; $R = 0.39$; $p < 0.01$, regression analysis).

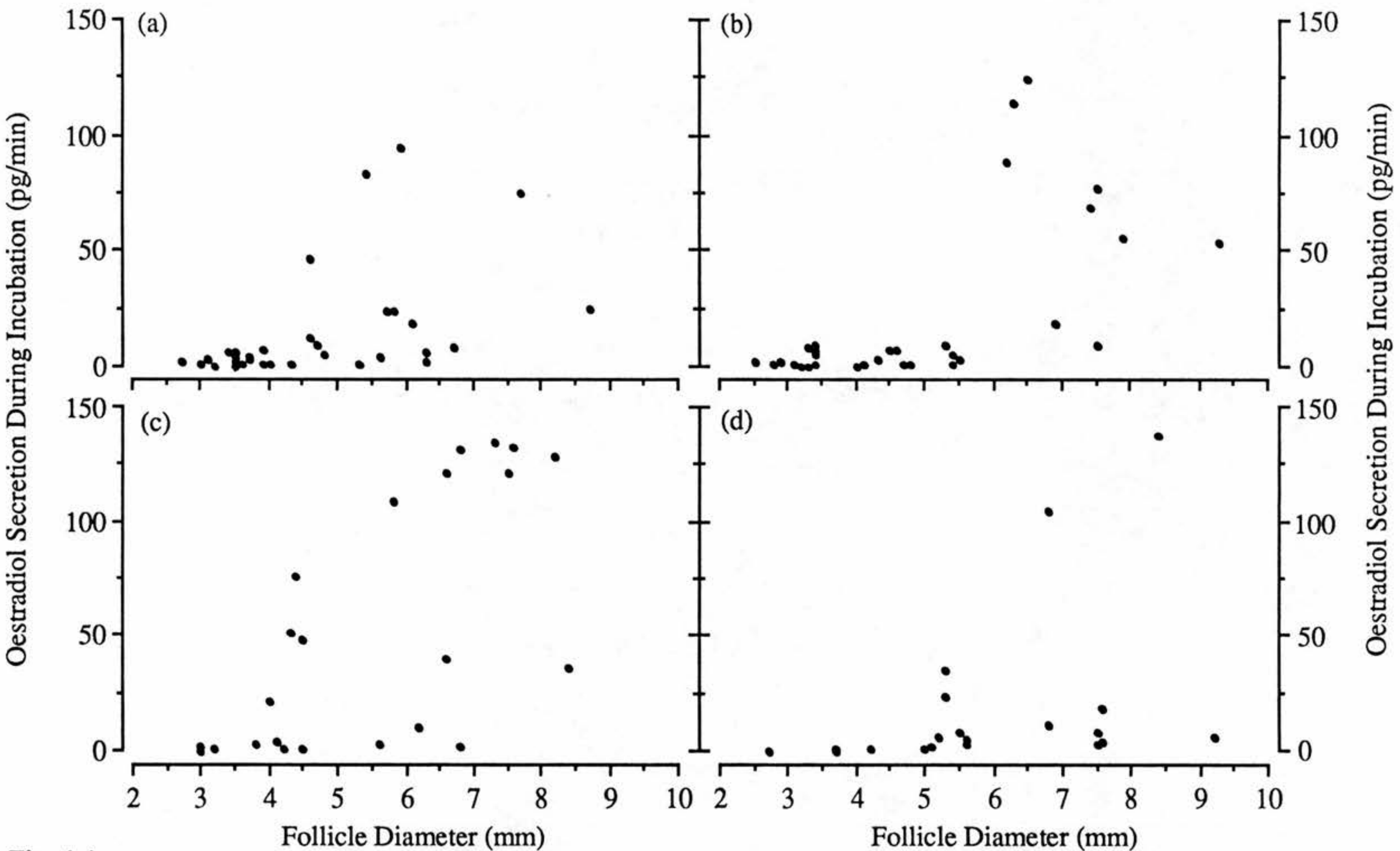

Fig. 4.4.
Relationship between follicle diameter and *in vitro* oestradiol secretion from follicles dissected from the ovaries of groups of 6 animals ovariectomised on a) day 10 of the luteal phase (n = 34) or during the follicular phase b) 24 - 30h (n = 33), c) 36h (n = 23) or d) 60h (n = 23) following an i.m. injection of 125µg cloprostenol on day 10 of the luteal phase.

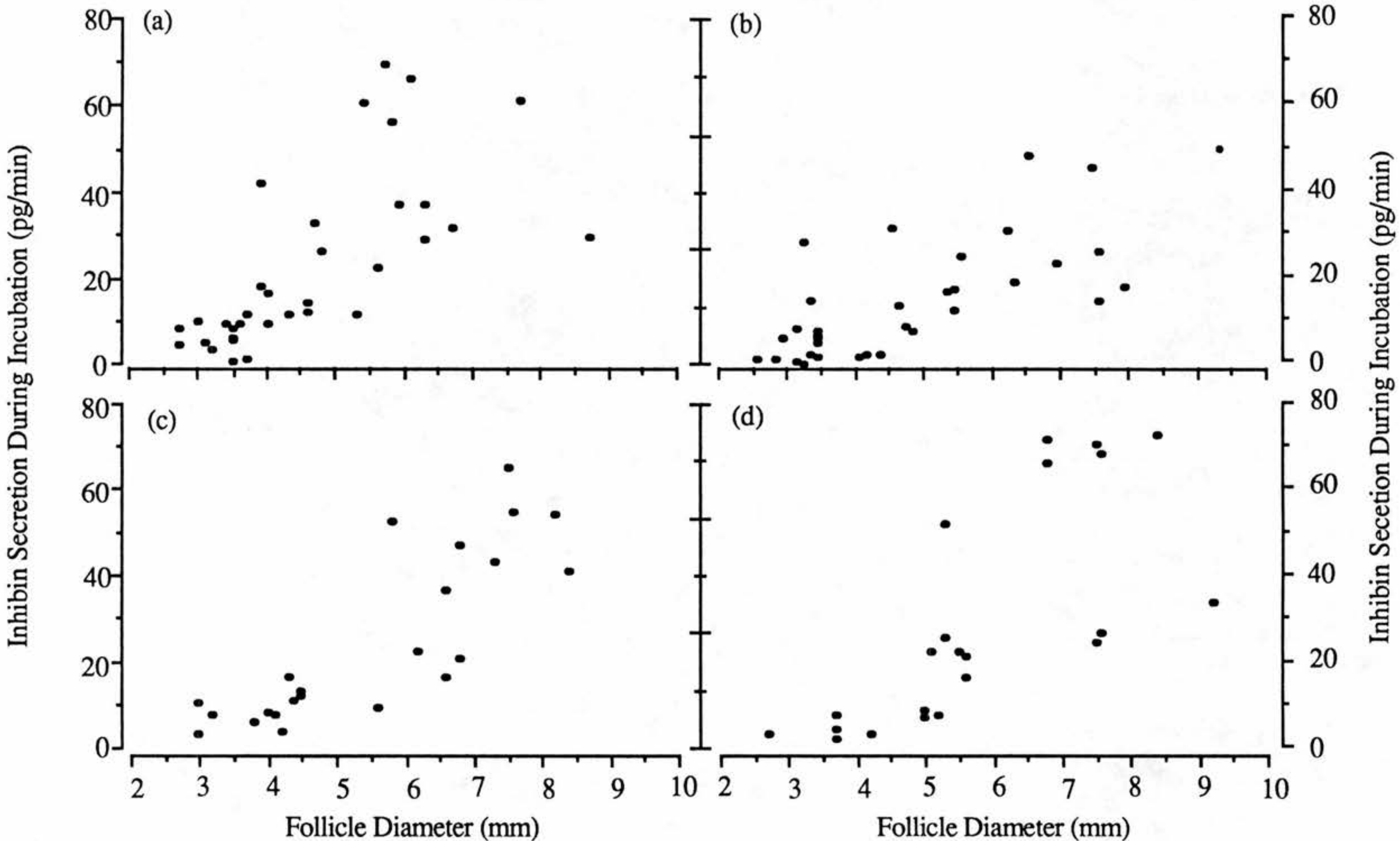

Fig. 4.5.

Relationship between follicle diameter and *in vitro* inhibin secretion from follicles dissected from the ovaries of groups of 6 animals ovariectomised on a) day 10 of the luteal phase (n = 34) or during the follicular phase b) 24 - 30h (n = 33), c) 36h (n = 23) or d) 60h (n = 23) following an i.m. injection of 125μg cloprostenol on day 10 of the luteal phase.

groups ($p < 0.001$) as well as the 24h group ($p < 0.05$), which in turn contained significantly more of these follicles than the luteal phase and 60h groups. This pattern of follicle oestrogen production was not mimicked by the production of inhibin, as when *in vitro* inhibin production was compared to follicle diameter a similar distribution of production in relation to follicle diameter was seen in all groups (Fig 4.5.).

The numbers of each class of follicle per ewe, in each of the 4 groups, are shown in Fig 4.6. The total number of follicles was lower in the two groups 36 and 60 h into the follicular phase though this difference was not significant. There were, however, significantly ($p < 0.05$) fewer small follicles in these two groups than in the luteal phase group or the 24h group. While the total number of large follicles did not differ between the four groups, there were significantly more large oestrogenic follicles in the 24h ($p < 0.05$) and 36h ($p < 0.01$) groups than in the luteal phase or 60h groups. The 36h group also contained significantly ($p < 0.05$) more large oestrogenic follicles than the 24h group, but possessed significantly ($p < 0.05$) fewer large non - oestrogenic follicles than any of the other groups.

Large oestrogenic follicles contained a 20 fold higher follicular fluid concentration of oestradiol than either large non - oestrogenic or small follicles (Fig 4.7.). Despite having similar low follicular fluid oestradiol concentrations the large non - oestrogenic follicles secreted significantly ($p < 0.01$) more oestradiol during incubation than the small follicles (8.0 ± 1.4 compared with 2.6 ± 0.4 pg/min) (Fig 4.8.). Like follicular fluid concentration, oestradiol production during incubation was also significantly ($p < 0.001$) higher from large oestrogenic follicles than from the other two groups. The concentration of inhibin in the follicular fluid of large oestrogenic follicles (1380 ± 105 ng/ml) was significantly higher than that of large non - oestrogenic or small follicles (Fig. 4.7.). However, in contrast to their very low oestradiol concentrations, these large non - oestrogenic and small follicles still contained appreciable concentrations of inhibin (676 ± 87 and 488 ± 62 ng/ml respectively). The production of inhibin during incubation was also greater ($p < 0.01$) in large oestrogenic follicles than in the other two groups (Fig 4.7.). While follicular fluid inhibin concentration did not differ significantly between large non - oestrogenic and small follicles, inhibin production during incubation was significantly ($p < 0.001$) greater from the larger follicles. Androstenedione concentration was significantly ($p < 0.05$) higher in the antral fluid of large oestrogenic follicles than non - oestrogenic ones, while the follicular fluid concentration in small follicles did not differ from that in either class of large follicle (Fig 4.7.). Androstenedione production during incubation was also higher ($p < 0.01$) from large oestrogenic than from large non - oestrogenic

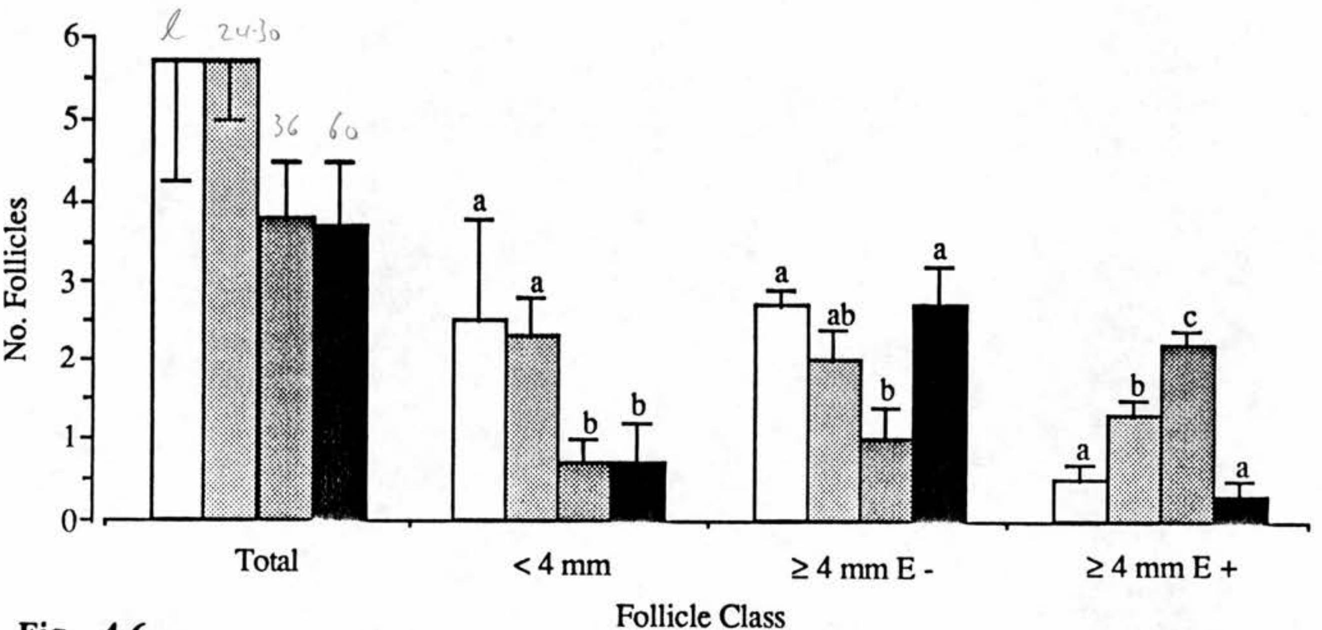

Fig. 4.6.
Mean (± S.E.M) total number of follicles (Total) or number of small (< 4 mm), large non - oestrogenic (≥ 4 mm E -) or large oestragenic (≥ 4 mm E+) follicles dissected from the ovaries of groups of 6 ewes ovariectomised on day 10 of the luteal phase (□) or during the follicular phase 24 - 30 (▨), 36 (▩) or 60h (■) following cloprostenol - induced luteal regressionon on day 10 of the luteal phase. Columns with different letters differ significantly; ab, bc $p < 0.05$; ac $p < 0.01$ (analysis of variance).

follicles (Fig 4.8.), while in contrast to antral fluid concentration, androstenedione production from small follicles was significantly lower than from both large non - oestrogenic ($p < 0.05$) and large oestrogenic ($p < 0.001$) follicles.

At the time of operation, the ovaries of the ewes contained a mean of 1.8 ± 0.1 corpora lutea per ewe indicating the ovulation rate in the previous cycle.

4.4. Discussion

In this study we have used hormone production over a 2h incubation period as an indication of the secretory capacity of individual follicles. When the secretion rate into the ovarian vein draining an ovary was compared to the combined *in vitro* hormone production from all the follicles dissected from that ovary, a good correlation was found for both inhibin and oestradiol. While the rate of secretion measured *in vivo* was some 20 to 30 fold higher than that seen during incubation, these high correlations do indicate that the incubation procedure was giving a reasonable indication of the pattern of secretory capacity of individual follicles. A lower production rate during the incubation procedure used in this study would, however, be expected due to the lack of gonadotropic stimulation and the absence of capillary blood flow. These results are in agreement with those of Webb & Gauld (1985) who showed that a similar 2h incubation reflected *in vivo* steroid production, as well as follicular fluid steroid content (Webb & Gauld, 1987).

There was a good correlation between the inhibin production *in vitro* and the diameter of the follicle. However, when a similar comparison was made for oestradiol the correlation was somewhat lower. This was due to the presence of a number of large follicles with low oestradiol production, i.e. the population of large atretic follicles which were still producing considerable quantities of inhibin. While large non - oestrogenic follicles were found to have lower follicular fluid inhibin concentrations and secrete less inhibin during culture than large oestrogenic follicles, the magnitude of these differences was much lower than was the case for oestradiol (2 fold for inhibin compared with 10 to 20 fold for oestradiol). The results of this study also revealed that small follicles provided a larger proportion of the total inhibin production than that of oestradiol (12% of inhibin production compared with 3% of oestradiol production), as did large non - oestrogenic follicles (33% compared with 7%). This resulted in large oestrogenic follicles contributing only 55% of the total inhibin production while contributing 90% of the total oestradiol production. The more even distribution of inhibin production between different follicle types parallels the lack of variation in

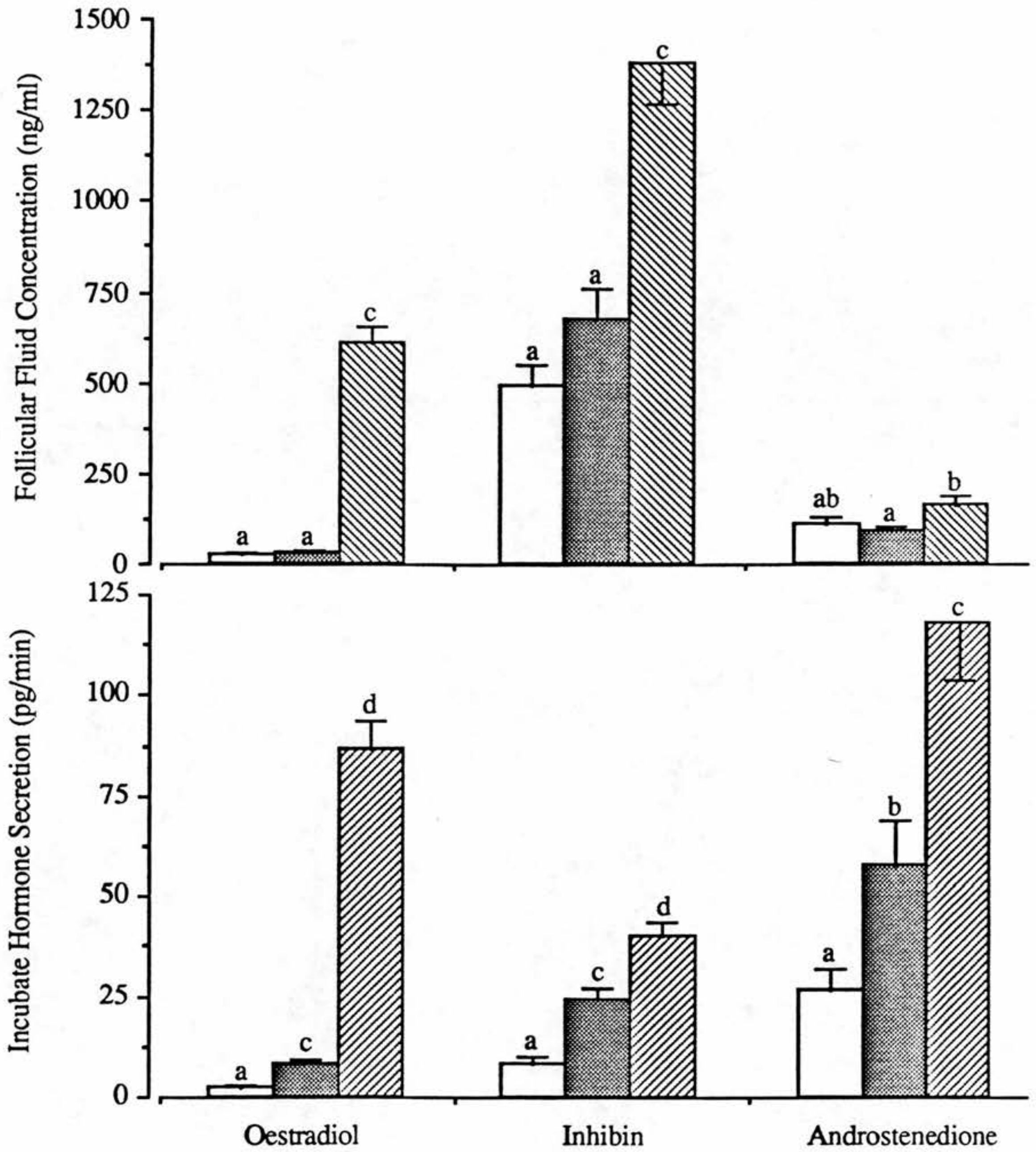

Fig. 4.7.
Mean (±S.E.M.) (a) concentration in the follicular fluid, and (b) secretion rate during incubation of oestradiol, inhbin and androstenedione from small (< 4 mm; n = 36, □) follicles and large (≥ 4 mm) non - oestrogenic (n = 49, ▩) and oestrogenic (n = 26, ▨) follicles. Columns with different letters differ significantly; ab $p < 0.05$, ac, bc, ($p < 0.01$ (analysis of variance).

inhibin secretion seen *in vivo* between groups of ewes with widely differing follicle populations in terms of follicle oestradiol production.

When oestradiol production from follicles was examined separately in the 4 groups of ewes at different stages of the oestrous cycle (Fig. 4.4.) more oestrogenic follicles were found in the ewes in the early and mid follicular phase groups than in the luteal phase or late follicular phase groups. This pattern results from the selection of ovulatory follicles early in the follicular phase, which then produce large quantities of oestradiol in response to increasing LH pulse frequency (Baird, 1978). By 60h an LH surge has occurred in most animals, which results in a fall in oestradiol production (Baird et al., 1981), hence there were a low number of oestrogenic follicles in this group. This pattern of follicle development is able to explain the elevation in the ovarian secretion rate of oestradiol seen in the early and mid follicular phase groups *in vivo*. When the same comparison was made for inhibin production (Fig 4.5.), a similar number of follicles producing large amounts of inhibin were found in the groups of ewes at all four stages of the oestrous cycle. This lack of change in the pattern of follicle inhibin production is in keeping with the relatively small differences in the ovarian secretion rate of inhibin *in vivo* between the four groups at different stages of the oestrous cycle. In the group operated on 36h following the induction of luteal regression there was an average of 2.0±0.4 large oestrogenic follicles per ewe, which corresponds closely to the ovulation rate value of 1.8± 0.1 obtained at surgery by counting the number of corpora lutea present. As the large oestrogenic follicles present at this time are the ones that will go on to ovulate, the similarity between these two values indicates that the definition of an oestrogenic follicle adopted in this study was an appropriate one.

It has been suggested that large "dominant" preovulatory follicles release oestradiol which feeds back on the pituitary resulting in a fall in FSH secretion which prevents development in the other less advanced follicles (Baird & McNeilly, 1981; Baird, 1983). Inhibin is also produced by the dominant follicle(s) and may be involved in the control of FSH secretion at this time. However, due to the relatively low proportion of inhibin production seen from the large preovulatory follicles compared with that of oestradiol (55% compared with 90%) and the lack of a rise in rate of ovarian inhibin secretion *in vivo* at this time it would seem that inhibin does not have a major role in this aspect of the follicle selection process. It has been suggested that inhibin may have an intra - gonadal role in the process of follicle selection and dominance (Ireland, 1987) the lack of a consistent rise in the peripheral concentration of FSH during the increase in ovulation rate seen following active immunisation against inhibin supporting this idea (Henderson et al., 1984). Once again the relatively low

proportion of inhibin production by the large preovulatory follicles and the fairly high inhibin production by atretic follicles shown in this study would suggest that inhibin does not play a major intra - gonadal role in the follicle selection/dominance process.

From the results of this study we conclude that unlike oestradiol, where most of the hormone is secreted by the large oestrogenic preovulatory follicles, a considerable amount of inhibin is also produced by large non - oestrogenic atretic follicles, and by small follicles, suggesting inhibin does not play an important role in follicle selection. The results also show that the lack of variation in the inhibin secretion rate in the intact animal is parallelled by a similar lack of variation in the pattern of inhibin production from individual follicles.

Chapter 5

Effect of Ovine Follicular Fluid on the Oestradiol Benzoate Induced LH Surge in Ovariectomized Ewes

5.1. Introduction

It is well documented that both ovine follicular fluid (oFF) and bovine follicular fluid (bFF) can suppress the concentration of FSH in sheep (oFF: Findlay et al., 1985; Clarke et al., 1986. bFF: McNeilly, 1984; Martin et al., 1987). The effects of oFF inhibin on LH are, however, far less clear. Suppression of FSH production has been shown by doses of follicular fluid containing quantities of inhibin similar to normal ovarian output (Martin et al., 1987), while suppression of LH has only been shown by administering large pharmacological doses of follicular fluid inhibin, well in excess of normal ovarian output. Pharmacological doses of oFF (Findlay et al., 1985; Clarke et al., 1986) and bFF (Martin et al., 1987) have been demonstrated to cause a reduction in mean LH levels by lowering pulse amplitude while not affecting pulse frequency. However, these effects are not consistent, as other studies involving large doses of follicular fluid inhibin have failed to exhibit any effects on LH (McNeilly, 1984; McNeilly, 1985; Al-Obaidi et al., 1986) or have shown an increase in mean LH concentration (Martin et al., 1986; Wallace & McNeilly, 1986). Scott & Burger (1981) showed that inhibin from ovine testicular lymph did not affect LH production from dispersed rat pituitary cells while Tsonis et al., (1986) showed that inhibin from oFF suppressed the production of FSH by dispersed ovine pituitary cells while not affecting LH production, even at high doses. This suggests that inhibin does not affect production of LH by the pituitary, even at pharmacological doses. While follicular fluid inhibin does not appear to affect basal LH production *in vitro*, there is evidence for the existence of a gonadotrophin surge-inhibiting factor in porcine follicular fluid (pFF), distinct from inhibin, which has been shown to inhibit the GnRH-stimulated release of FSH and LH from rat pituitary cell cultures, while not affecting basal LH concentrations (Danforth et al., 1987). pFF has also been shown to exhibit gonadotrophin surge-inhibiting activity *in vivo*, blocking the positive feedback effects of oestradiol benzoate in generating LH surge release in both entire (Hodgen et al., 1980) and castrate (Sopelak & Hodgen, 1984) female monkeys.

In this study we investigated the effects of oFF on oestradiol benzoate-induced gonadotrophin responses in ovariectomised ewes in order to study further the role of

inhibin in the control of gonadotrophin release, and also to investigate the possibility of the presence of a gonadotrophin surge-inhibiting factor in oFF.

5.2. Materials and Methods

5.2.1. Purification Procedure

Immunopurification of inhibin was performed using an affinity gel composed of an ovine anti porcine 1 - 26α inhibin antiserum (S55) bound to cyano-bromide activated sepharose - 6B (Pharmacia) (McNeilly et al., 1989). One hundred ml from a pool of ovine follicular fluid (oFF) which had been charcoal - stripped and passed through C18 Sep-pak columns (Waters Associates, Milford, MA, USA) to remove steroids was centrifuged to remove particulate matter, passed down a 70ml inhibin affinity gel column 5 times and then frozen for use as an oFF treatment with lowered inhibin content. The column was then washed with 140 ml (2 x column volume) of 0.05M Tris HCL buffer (pH 7.5) containing 0.05% CHAPS (Tris buffer), followed by 140ml Tris buffer containing 2 mol NaCl/l. The immunopurified inhibin was then eluted from the column using Tris buffer containing 8 mol urea/l. The inhibin was then dialysed for 24 hrs against 4 changes of 5l deionized water at room temperature. The oFF inhibin was then lyophilysed and reconstituted in 5ml Tris buffer. The pooled oFF had a protein content of 46.8 mg/ml and an inhibin concentration of 2.8 μg/ml. The partially purified inhibin was made up to 100ml before injection giving protein and inhibin contents of 1.9 mg/ml and 0.8 μg/ml. The oFF which had been passed down the column had a protein content of 18.6 mg/ml and an inhibin content of 0.06 μg/ml.

5.2.2. Experimental Procedure

The experiment was performed in 23 mature Scottish Blackface ewes which had been ovariectomised 8 weeks previously. Animals were allocated to one of four groups with similar mean group weights (58±2kg).

1 - Saline (Control; n=6)
2 - Steroid-stripped ovine follicular fluid (oFF; n=6)
3 - Partially purified inhibin (Inhibin; n=6)
4 - Inhibin with lowered inhibin content (Low Inhibin oFF; n=5)

One ml of saline or test substance was injected subcutaneously at 8h intervals throughout the 40h experimental period, and 50μg oestradiol benzoate injected i.m. 8h into the experiment to induce an LH surge. Prior to treatment jugular veins were cannulated using plastic cannulae with three-way taps and 5 ml samples of jugular venous blood collected at hourly intervals throughout the experiment.

5.2.3. Analysis of Data

The suppression of LH following the injection of oestradiol benzoate was analysed by analysis of variance, while the timing and magnitude of the LH surge were compared between groups by means of a Students unpaired t-test on untransformed data. The FSH decline following oestradiol benzoate administration was analysed between groups using analysis of variance and Duncan's multiple range test on data that had been transformed to a percentage of the mean concentration during the 8h period prior to oestradiol benzoate administration in order to overcome differences in FSH concentrations between groups. The maximum degree of FSH suppression was determined by comparing the FSH concentration from 36 - 40h, during which time no further suppression of FSH occurred, with the concentration prior to oestradiol benzoate injection.

5.3. Results

Following ovariectomy the mean concentration of inhibin in the jugular venous plasma of the experimental animals fell from a mean of 76 ± 7 pg/ml to below the sensitivity of the assay (< 20 pg/ml). In all three treatment groups the concentration of inhibin was increased following the injection of test substance (Fig. 5.1). Treatment with oFF generated a mean inhibin concentration, during the period from 8h to 40h, of 112.5 ± 9.6 ng/l which was significantly ($p < 0.001$) higher than that seen in the group injected with the inhibin preparation (63.4 ± 5.3). In the group injected with the low inhibin oFF the mean concentration of inhibin was 36.9 ± 1.8, however in the animals in this group concentrations of inhibin were not consistently maintained above the sensitivity of the assay.

Following the administration of oestradiol benzoate all 4 groups exhibited a marked suppression in the plasma concentration of LH ($p < 0.001$), this suppression not differing significantly between the 4 groups (Fig. 5.2). This suppression of LH was then followed by an LH surge in all animals. In the control group this surge was

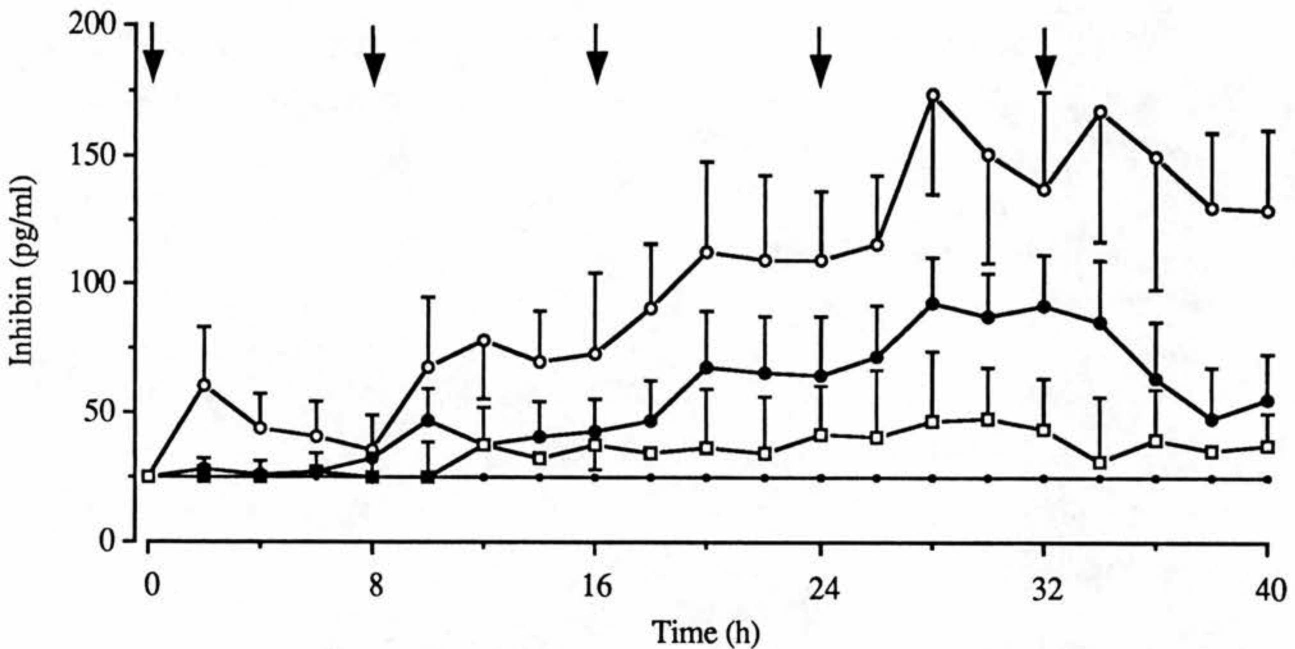

Fig. 5.1.
The mean concentration of inhibin in the jugular venous plasma of animals injected at 8h intervals with; control saline (•; n = 6), oFF (o; n = 6), a partially purified preparation of inhibin (● ; n = 6) or oFF with lowered inhibin content (□;n = 5), and injected at 8 h with a single i.m. injection of oestradiol benzoate (E2).

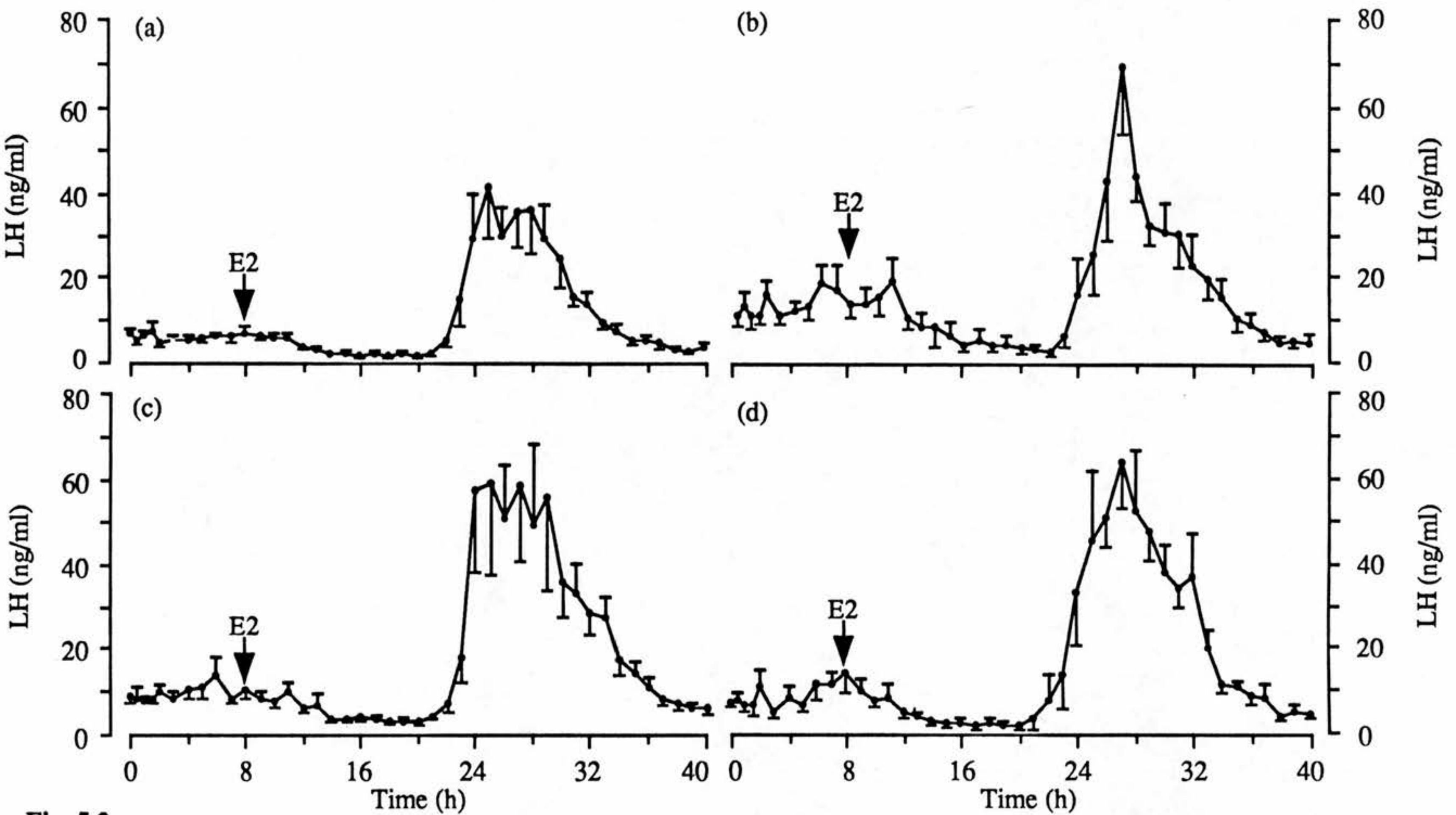

Fig. 5.2.
The mean jugular venous plasma concentration of LH in ewes injected subcutaneously at 8 h intervals with a) control saline (n = 6), b) oFF (n = 6), c) a partially purified inhibin preparation (n = 6) or d) oFF with lowered inhibin content, and injected at 8 h with a single i.m. injection of 50 µg oestradiol benzoate (E2).

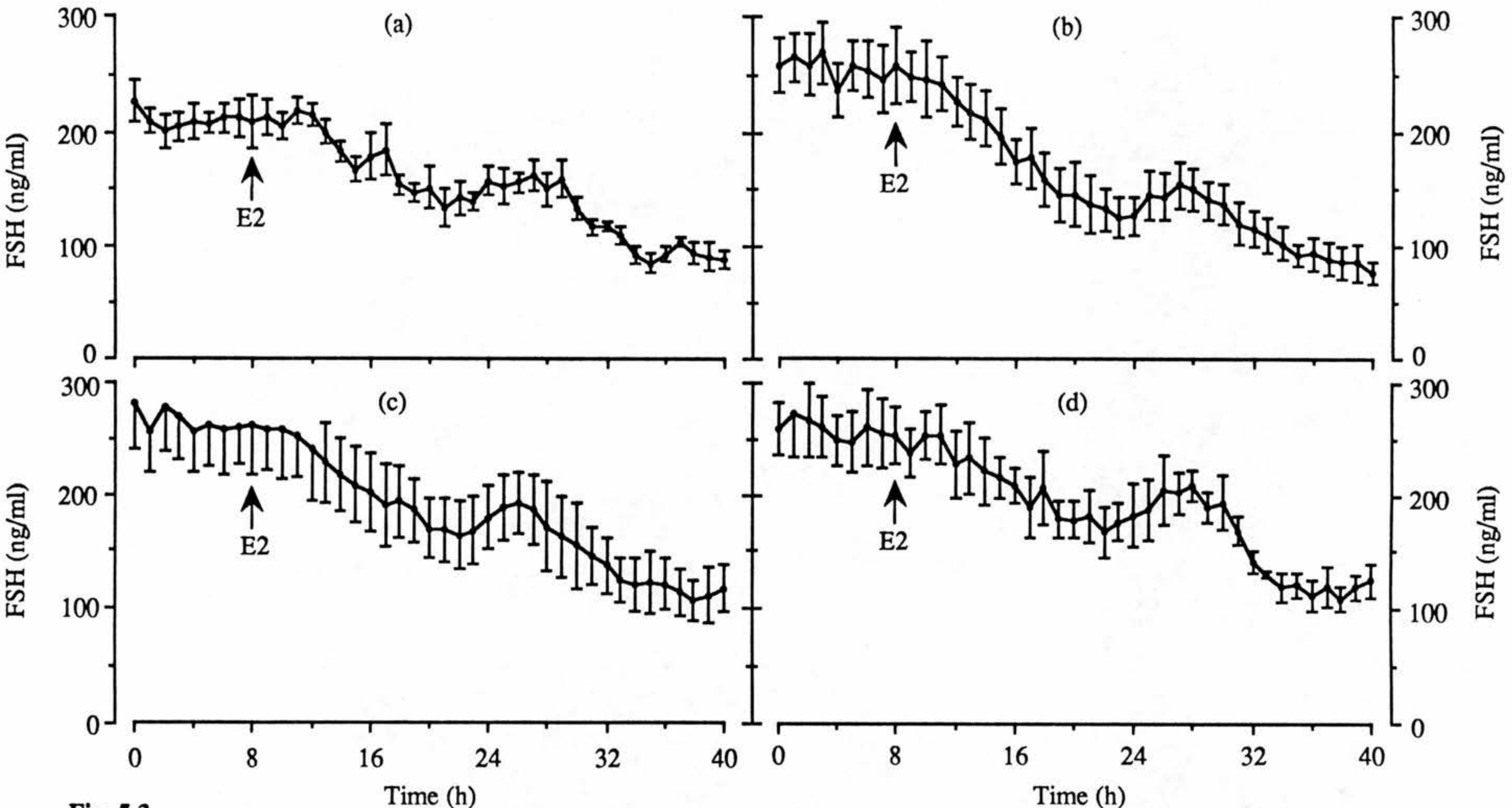

Fig. 5.3.
The mean jugular venous plasma concentration of FSH in ewes injected subcutaneously at 8 h intervals with a) control saline (n = 6), b) oFF (n = 6), c) a partially purified inhibin preparation (n = 6) or d) oFF with lowered inhibin content, and injected at 8 h with a single i.m. injection of 50 μg oestradiol benzoate (E2).

generated by 15.2±0.6h, reaching a peak of 57.0±11.0μg/l by 17.7±0.6h and lasting 8.3±1.2h. In the three treatment groups the characteristics of the LH surge showed no significant difference from the control group (Table 5.1).

In all 4 groups FSH showed a significant ($p < 0.001$) decline following the injection of oestradiol benzoate, with a small rise, concurrent with the LH surge, being seen in all groups by about 16h following oestradiol benzoate administration (Fig 5.3).

Table 5.1. The effect of treatments on the characteristics of the LH surge.

Group	Time to Surge (h)	Peak Height (ng/ml)	Time to Peak (h)	Surge Duration (h)
Control	15.3 ± 0.6	57.0 ± 11.0	17.7 ± 0.6	8.3 ± 1.2
oFF	16.5 ± 0.4	72.5 ±15.9	19.3 ± 0.8	8.8 ± 1.5
Inhibin	16.0 ± 0.8	94.2 ± 21.5	18.7 ± 1.2	10.3 ± 1.0
Low Inhibin oFF	15.6 ± 0.8	77.6 ± 8.9	18.4 ± 0.9	10.8 ± 0.9

While during the 8h period prior to oestradiol administration, there was no change in the concentration of FSH in any group ($p > 0.5$), the mean concentration of FSH was significantly ($p < 0.05$) lower in the control group (210±12) than in any of the treatment groups (oFF 257±24; inhibin 278±30; low inhibin oFF 261±27). To allow the comparison of the degree of FSH suppression between groups with differing initial FSH concentrations, the level of FSH suppression was therefore converted to a percentage of the concentration during this period prior to oestradiol benzoate administration Fig 5.4. The mean and maximum levels of FSH suppression in the four groups are shown in Table 5.2.

Table 5.2. The effects of treatments on the degree of FSH suppression.

Group	Mean FSH Suppression (%)	Max FSH Suppression (%)
Control	76.3 ± 3.2	45.2 ± 2.6
oFF	66.8 ± 3.9 **	28.4 ± 4.0 ***
Inhibin	73.9 ± 3.2	42.0 ± 2.0
Low Inhibin oFF	77.4 ± 3.0	45.1 ± 2.4

(** $p < 0.01$) (*** $p < 0.001$)

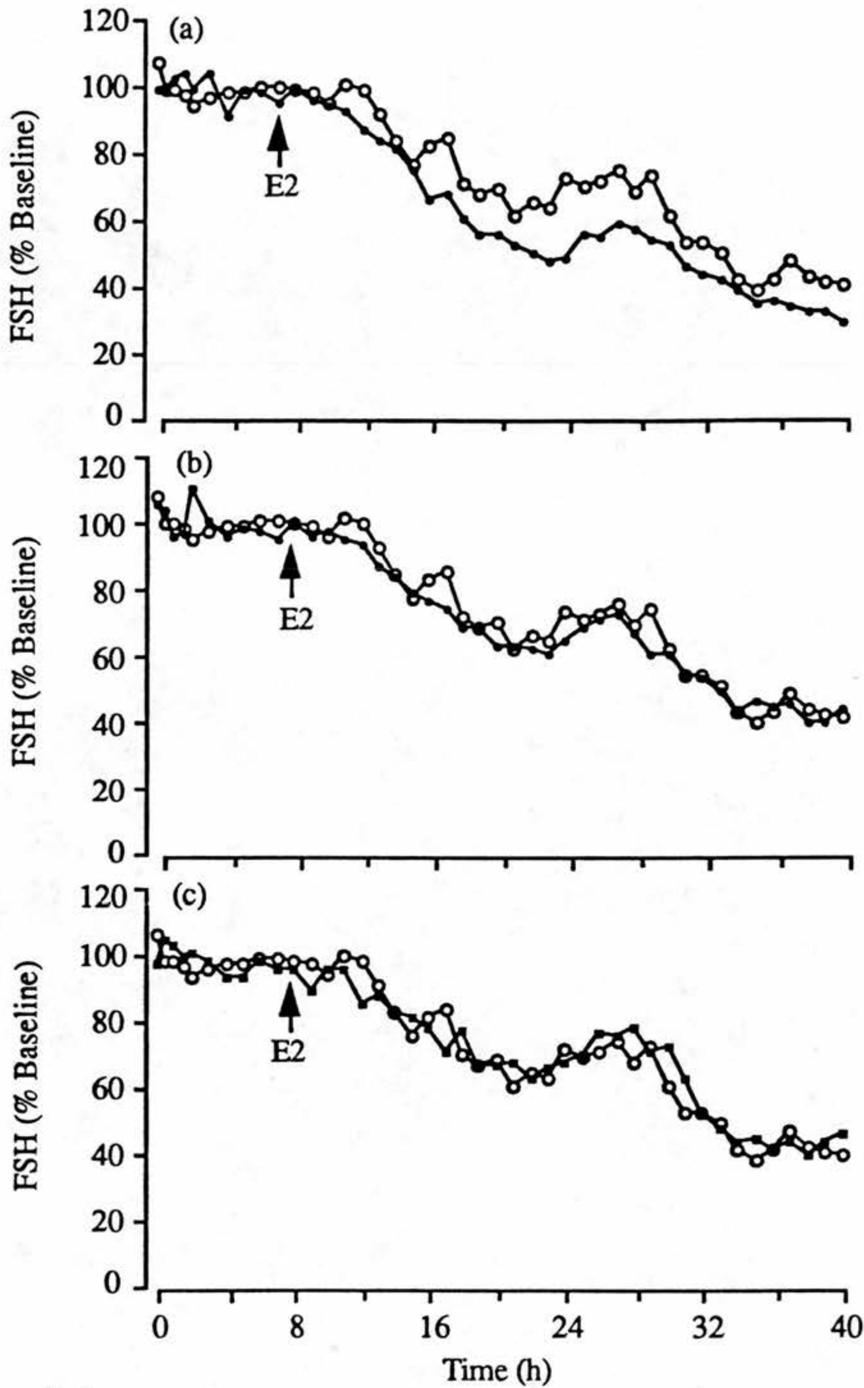

Fig. 5.4.
The mean degree of suppression of plasma FSH concentration in control ewes (•) compared to treated ewes (o) injected at 8 h intervals with a) oFF (n = 6), b) a partially purified inhibin preparation (n = 6) or c) oFF with lowered inhibin content, and injected at 8 h with a single i.m. injection of 50 µg oestradiol benzoate (E2).

The mean suppression over the response period was significantly greater in the oFF-treated group than the control group (66.8±3.9 v 76.3±3.2% of pre-treatment levels; $p < 0.01$), as was the maximum degree of suppression (28.4±4.0 v 45.2±2.6 % of pre-treatment levels; $p < 0.001$). The groups treated with inhibin and low inhibin oFF showed no significant differences from the control group in the mean or maximum degree of FSH suppression.

5.4. Discussion

In this study we have shown that a dose of follicular fluid sufficient to generate peripheral plasma inhibin concentrations in ovariectomised ewes only slightly higher than concentrations found prior to ovariectomy, while causing a significant suppression of FSH concentration, resulted in no significant changes in the characteristics of an oestradiol benzoate - induced LH surge. This result adds weight to the definition of inhibin as a selective suppressor of FSH, while providing further evidence that inhibin does not play a role in the regulation of LH production.

In a study performed on the same breed of sheep Martin et al., (1987) concluded that a dose of 0.6ml bFF every 8h delivered quantities of inhibin approximately equivalent to normal ovarian output. The bFF used in that study had an inhibin content, measured in a sheep pituitary cell bioassay (Tsonis et al., 1986), of 13.4kU/ml which works out at a dose rate of 8kU inhibin bioactivity per 8h. In this study we used a dose of 1ml of oFF every 8h with a bioactivity, measured in the same assay, of 8.7kU/ml. Our slightly higher dose of inhibin generated peripheral inhibin concentrations higher than those seen prior to ovariectomy and so it would seem that a dose a little less than 8kU/8h would be appropriate to restore normal inhibin concentrations in this breed of sheep following ovariectomy.

In the group treated with oFF no decline in the concentration of FSH was seen following the first injection, the decline in FSH not being evident until after the injection of oestradiol benzoate 8h into the experiment. Delays in the response of FSH following i.v. injection of follicular fluid of 2 - 3h (McNeilly, 1984; Wallace & McNeilly, 1986) and 4h (Tsonis et al., 1986) have been reported, as has a loss of pituitary responsiveness to inhibin with time following ovariectomy (Martin et al. 1987). These factors, together with the fact that in this study a subcutaneous route of oFF administration was used are therefore probably sufficient to account for the delay in response seen in this study.

The fact that the low inhibin oFF did not cause any suppression of FSH concentration over that produced by the oestradiol benzoate suggests that it was the inhibin in the oFF that was producing the suppression of FSH in the oFF group. This conclusion is confounded by the fact that in the animals injected with the partially purified inhibin preparation there was no suppression of FSH over that produced by the administration of oestradiol benzoate. This was probably due to the use of too low a dose of inhibin to exert a detectable effect on FSH concentration in the presence of the high dose of oestradiol. In this group the peripheral concentration of inhibin was only half that seen in the group treated with the neat follicular fluid, and inhibin concentrations remained significantly lower than those seen prior to ovariectomy until 20h into the experiment.

In monkeys porcine follicular fluid has been demonstrated to block oestradiol benzoate - induced LH surges in both intact (Hodgen et al., 1980) and ovariectomised (Sopelak & Hodgen, 1984) animals and the substance responsible for this activity has now been isolated (Danforth et al., 1987). The fact that in this study the oFF and the low - inhibin oFF did not affect the timing, magnitude or duration of the LH surge would indicate that in the sheep, follicular fluid does not contain a gonadotrophin - surge - inhibiting factor as appears to be the case in pFF.

From the results of this study we conclude that inhibin in oFF acts to suppress FSH production alone, and does not affect the production of LH. Furthermore we found no evidence for the existence of a "non-steroidal" factor in the follicular fluid of this species responsible for the modulation of the LH surge.

Chapter 6

Passive Immunisation against Inhibin during the Luteal Phase of the Oestrous Cycle

6.1. Introduction

While it is now well established that inhibin has the ability to suppress follicle stimulating hormone (FSH) production by the anterior pituitary gland both *in vitro* and *in vivo*, the physiological role of inhibin remains uncertain. Inhibin in charcoal-stripped follicular fluid causes suppression of the production of FSH (McNeilly, 1985; Wallace & McNeilly, 1985) and large doses of follicular fluid can suppress the concentration of FSH in normal ewes to undetectable levels (Martin et al., 1987), indicating the potential power of inhibin as a regulator of FSH. However, doses of inhibin estimated as approximately equivalent to normal ovarian output produce only transient falls in the concentration of FSH (Martin et al., 1987). Oestradiol will also suppress the production of FSH, but is unable to restore fully the normal concentration of FSH when administered to acutely ovariectomized ewes (Goodman et al., 1981). There is now evidence that inhibin and oestradiol act synergistically in the regulation of FSH production by negative feedback on the anterior pituitary (Martin et al., 1988), but the relative importance of inhibin in this negative feedback system still remains unclear.

Antisera raised to inhibin partially purified from bovine follicular fluid (bFF) have been shown to neutralise the *in vitro* FSH-suppressive activity of inhibin in follicular fluid from a number of species (van Dijk et al., 1986). Active immunization of ewes against a subunit of bovine inhibin produced by recombinant DNA techniques (Forage et al., 1987; Findlay et al., 1989), a synthetic peptide fragment (Wrathall et al., 1989) or against partially purified preparations of inhibin from bovine follicular fluid (bFF) (Cummins et al., 1986; Henderson et al., 1984) all resulted in an increase in ovulation rate. However, in these studies no consistent increases in the concentration of FSH have been demonstrated. Antisera raised to inhibin from bovine follicular fluid have also been shown to neutralise inhibin activity *in vivo* in castrate ewes where passive immunization against inhibin was shown to reduce the FSH-suppressing effects of bFF (Al-Obaidi et al., 1986) and in rats where neutralisation of endogenous inhibin by an inhibin antiserum caused an increase in plasma concentration of FSH (Rivier et al., 1986; Culler & Negro-Vilar, 1988). By acutely perturbing the feedback mechanisms, passive immunization may permit a change in the concentration of FSH to

be detected before a compensatory increase in the secretion of inhibitory factors by the ovary has taken place.

In this study we have investigated the ability of an ovine antiserum, raised to a synthetic inhibin peptide fragment, to neutralise, *in vitro*, the suppressive effect of ovine inhibin on the production of FSH by dispersed cells from the anterior pituitary. We have then determined the optimum dose of this antiserum for passive immunization, and used this dose to neutralise circulating inhibin in intact ewes as a means by which to investigate the importance of inhibin in the control of the production of FSH in the luteal phase of the sheep oestrous cycle.

6.2. Materials and Methods

6.2.1. Inhibin Antiserum

The anti-inhibin antiserum used in this study was raised in an ovariectomized Scottish Blackface ewe (McNeilly et al., 1989) to a synthetic peptide of the 1-26 amino acid sequence of the N-terminus of the α chain of 32 kDa porcine (p) inhibin (Rivier et al., 1986) conjugated to ovalbumin. The final titre of this antibody, assessed at 50% binding of ^{125}I-labeled p1-26α peptide was 1: 40000.

6.2.2. *In Vitro* Inhibin Neutralisation

6.2.2.1. Pituitary Cell Cultures

Cultures of dispersed ovine pituitary cells from mature female sheep were produced by the method previously described for the inhibin bioassay. Aliquots of 50μl of cells (200000) were plated out with 550μl modified DMEM into individual wells of plastic culture plates (Costar; Flow Laboratories) and preincubated for 48h. The medium was then removed and replaced with varying dilutions of ovine follicular fluid (oFF) with or without inhibin antibody, and allowed to incubate for a further 48h. The incubate media were then collected and frozen at -20°C.

6.2.2.2. Neutralisation of Inhibin Activity

Inhibin activity was obtained by using a pool of ovine follicular fluid (oFF) which had been charcoal stripped (Tsonis et al., 1986) to remove steroids. The potency of this

oFF pool was 9000 U/ml, expressed against a stable lyophilized ovine rete testis fluid preparation with an arbitrary potency of 1U/mg (Eddie et al., 1979). Five dilutions of this oFF (containing 0.8 - 66.7nl oFF) were used to generate an FSH inhibition curve, expressed as a percentage of control FSH production with no oFF. This inhibition was then neutralised using the inhibin antibody (S40). Aliquots of 50nl of oFF diluted to a final volume of 50μl were preincubated with 50μl aliquots of 8 dilutions of inhibin antibody (0.078-10μl antibody/dilution) at room temperature for 1h. Antibody/oFF incubates were then added to the pituitary cell cultures in quadruplicate, made up to a final volume of 600μl with supplemented DMEM, and incubated for 48h. FSH concentration was then measured in all samples of culture medium by radioimmunoassay.

6.2.3. Pilot Dose Study

The dose trial was carried out during the breeding season in November on six mature (2-3 year old) Finn x Merino ewes. Oestrous cycles were synchronized and animals were then allocated to three groups of two animals with similar mean group weights of approximately 42kg. On Day 10 of the luteal phase animals were given an intravenous bolus injection of either 1ml, 5ml, or 10ml of inhibin antibody, and hourly peripheral 5ml plasma samples collected for 4h before and 16h after antibody administration. Sampling rate was then reduced to 4 hourly for 16h, and finally to 8 hourly until 48h after antibody administration when sampling ceased. Samples were then assayed by radioimmunoassay for FSH, and the inhibin binding capacity of the plasma determined.

6.2.4. Immunization Experiment

The experiment was carried out during the breeding season, in November and December, on 10 mature Scottish Blackface ewes. Oestrous cycles were synchronized and the animals were then allocated to two groups of five animals with similar mean group weights (Control: 54.6±3.0kg; immunised: 54.0±1.5kg; mean ± S.E.) and their jugular veins cannulated using plastic cannulae and three-way taps. Animals were then given an intravenous bolus injection on Day 8 of the luteal phase of the oestrous cycle of either 10ml of normal sheep plasma (Control) or 10ml of the inhibin antibody (immunised). Hourly 5 ml peripheral venous blood samples were collected for 4h before and 16h after administration of either antibody or control plasma. Samples were then collected 4 hourly for a further 16h, and then 8 hourly until 144h after treatment. On

day 10 of the luteal phase, 48h after antibody/plasma administration, all animals were injected i.m. with Cloprostenol (100µg) to induce luteal regression and hence ovulation. Animals were checked for oestrus by teaser ram, and six days after Cloprostenol injection laparoscopy was performed on all animals to determine ovulation rate. Blood samples were then assayed by radioimmunoassay for FSH, LH, and progesterone and the inhibin binding capacity in the plasma of immunised ewes determined.

6.2.5. Analysis of Data

The rise in FSH in response to immunization was defined to have commenced at the first point on a sequential FSH rise with an increase in concentration over the previous sample in excess of the sum of the assay error, and to have ended at the point where the concentration was no longer greater than pretreatment level by more than the assay error. The assay error was estimated from the the coefficient of variation determined in a plasma pool containing approximately the same concentration of FSH, and was defined as two times the standard deviation at that concentration. The maximum FSH response was defined to have started at the point when a sample was not followed by a rise in concentration greater than the sum of the assay error, and to have ended at the point on a sequential fall which was followed by a fall in concentration greater than the assay error.

Due to the small group size (n = 2) statistical analysis was not performed on the FSH responses in the pilot study. However, due to the variation in concentration of FSH between animals the results were expressed as a percentage of the baseline concentrations.

In the main experiment the response of FSH seen in the immunised group was compared to the control group by analysis of variance on repeated samples, and the concentrations of FSH in control and immunised animals, during the 48h period following injection of plasma or antiserum, compared by analysis of variance and Duncan's multiple range test. A Student's unpaired t-test was used to compare progesterone concentrations before and after injection of cloprostenol and to compare binding capacities at different times.

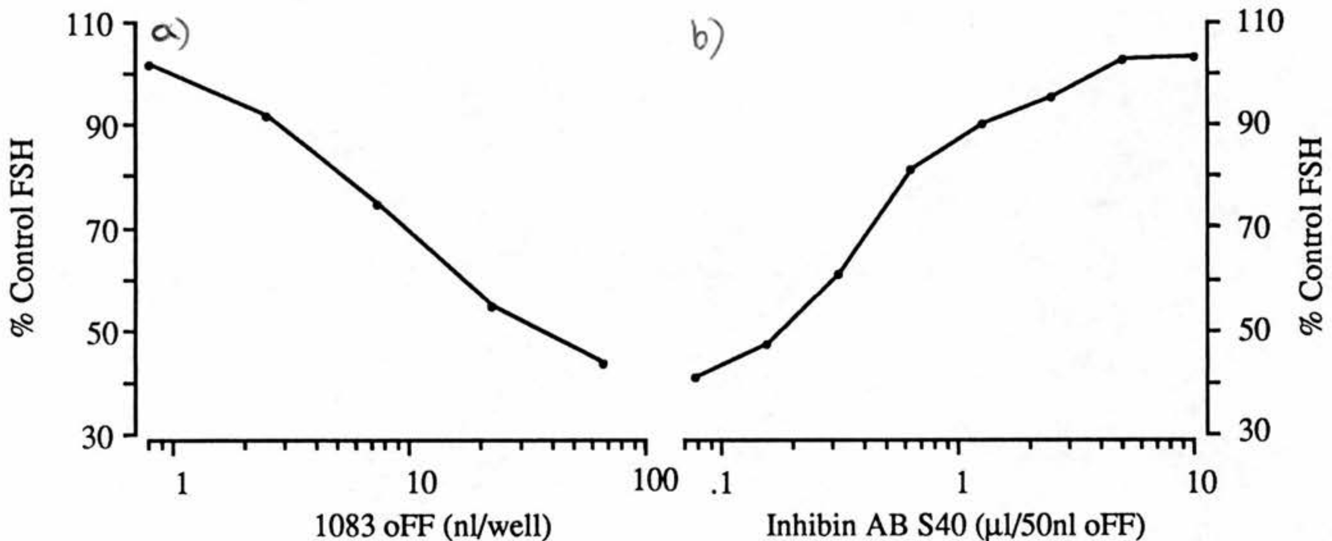

Fig. 6.1.
(a) Suppression of the production of FSH by ovine pituitary cells in culture using increasing amounts of charcoal stripped ovine follicular fluid (oFF). (b) Dose - dependent reversal of the effect of a maximally suppressing dose of oFF (50 nl) by increasing amounts of porcine 1 - 26α inhibin antiserum.

6.3. Results

6.3.1. *In Vitro* Neutralisation

From the FSH inhibition curve (Fig. 6.1.) it can be seen that 50nl oFF per well, used as the dose of inhibin for neutralisation by the antibody, produced an almost maximum degree of suppression (46% of control compared to 44% of control by 66.6nl oFF). It can also be seen from Fig. 6.1. that the inhibin antibody neutralised the suppression of FSH production by the inhibin in the oFF in a dose related manner, and was able to neutralise this inhibin activity completely.

6.3.2. Pilot Dose Study

The inhibin binding capacity generated in the plasma of immunised ewes, at a dilution of 1:15, is shown in Fig. 6.2. The 3 doses of antibody each generated a greater mean inhibin-binding capacity than the previous dose (1ml: 53.7%; 5ml: 64.4%; 10ml: 70.7%). All 3 doses showed a fall in binding capacity by 40h (1ml: 33.9 %; 5ml: 58.2 %; 10ml: 64.6 %), the 1ml dose resulting in the most marked fall and the 10ml group the least.

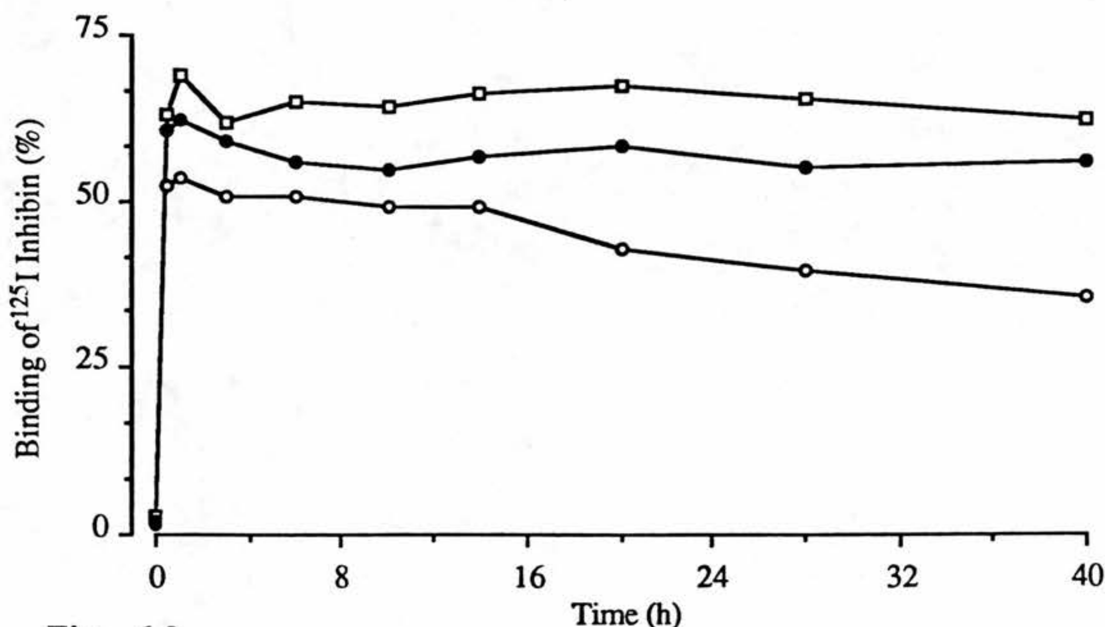

Fig. 6.2.
Responses in the inhibin - binding capacity of plasma at a dilution of 1:15 resulting from the injection at time 0 of an i.v. bolus of 1 (○), 5 (●) or 10 ml (□) inhibin antibody in ewes. (Values are means of duplicate determionations).

In all 3 groups immunization was followed by a rise in the concentration of FSH in the peripheral plasma (Fig. 6.3.). In the 1ml group FSH concentration rose from a mean of 1.61 ng/ml before treatment to a maximum of 2.40 ng/ml (mean 150%; range 133 to 167%), in the 5ml group this rise was from 1.68 to 2.87 ng/ml (mean 183%; range 131 to 235%) and in the 10ml group 2.27 to 4.48 ng/ml (mean 200%; range 167 to 232%). Due to the small group size, and the variable nature of the responses it is not possible to draw firm conclusions, but it would appear that, depending on animals, a maximum response to immunization can be achieved with between 5ml and 10ml of the antiserum. To ensure a maximum response of FSH to immunization a dose of 10ml was therefore used in the luteal phase immunization experiment.

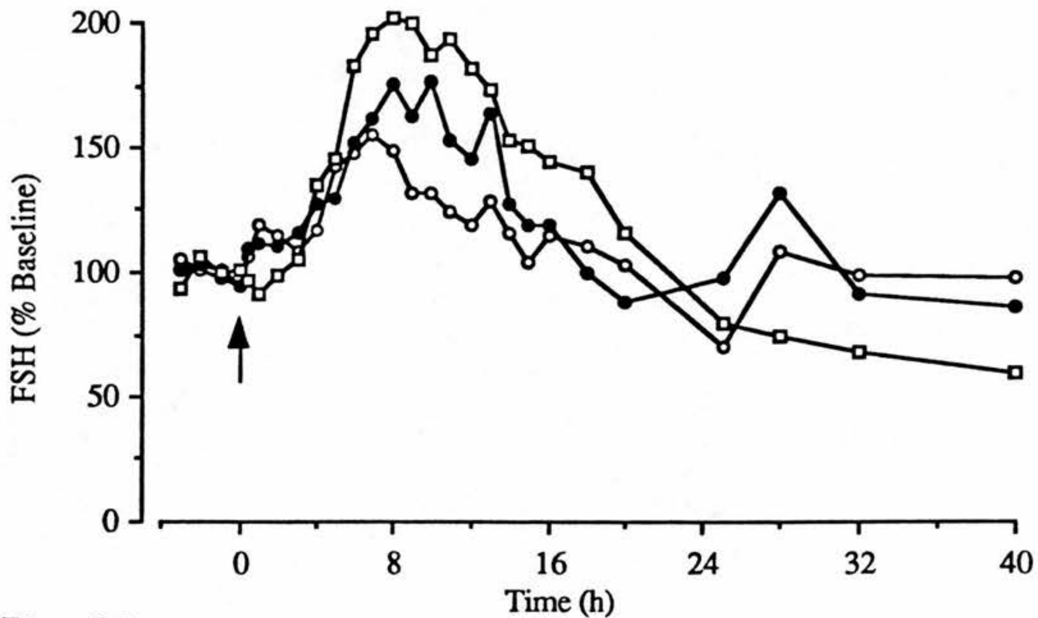

Fig. 6.3.
The plasma concentration of FSH as a percentage of the pretreatment baseline concentration resulting from the injection at time 0 of an i.v. bolus of 1 (○), 5 (●) or 10 ml (□) inhibin antibody in ewes. (Values are means of duplicate determionations).

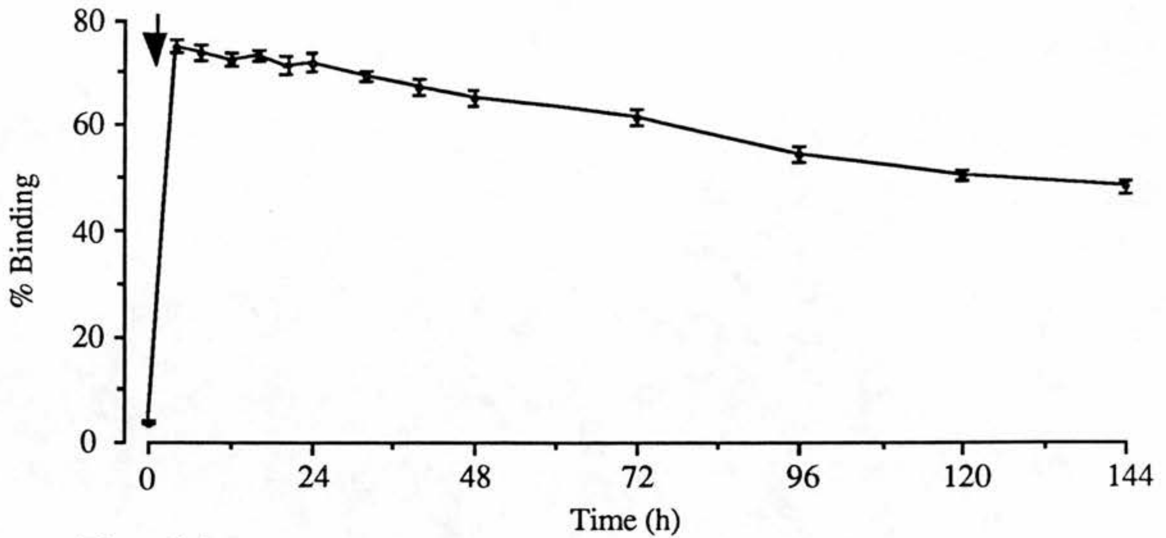

Fig. 6.4.
Mean (± S.E.M) inhibin binding capacity generated in the plasma, at a dilution of 1:15, of ewes injected at time 0 with a single i.v. bolus of inhibin antibody (n = 5).

6.3.3. Luteal Phase Immunization

Following immunization there was an increase in the ability of plasma to bind inhibin in all the immunised animals (Fig. 6.4.). At a dilution of 1:15 pre-immunization plasma exhibited a non specific binding of 3.5±0.3% while 1h after injection of antibody the same dilution of plasma bound 75.0±1.2%. By 48h binding had fallen to 65.1±1.2% ($p < 0.001$) and by the end of the experiment at 144h a further fall had taken place to 48.3±1.1% ($p < 0.001$).

Concentrations of progesterone in the plasma of both groups were similar during the 32h period following injection of plasma or antiserum (Fig. 6.5.; 2.54±0.32 ngl/ml in the control group compared with 2.52±0.26 ng/ml in the immunised group), neither group showing any response to treatment. Following cloprostenol-induced luteal regression the concentration of progesterone fell significantly ($p < 0.001$) in both groups by 16h, and by 32h following cloprostenol the concentration of progesterone in the peripheral plasma of all animals had fallen to below 0.1 ng/ml in both groups.

Administration of the normal sheep plasma or the antiserum to inhibin had no effect on the mean concentration of LH in peripheral plasma (Fig. 6.6.). In the control group the mean concentration LH was 0.73±0.02 ng/ml before treatment and 0.80±0.04 ng/ml during the 32h period following treatment. In the immunised group the corresponding concentrations of LH were 0.69±0.03 and 0.77±0.06 ng/ml. Following cloprostenol-induced luteal regression the time to the LH surge was the same

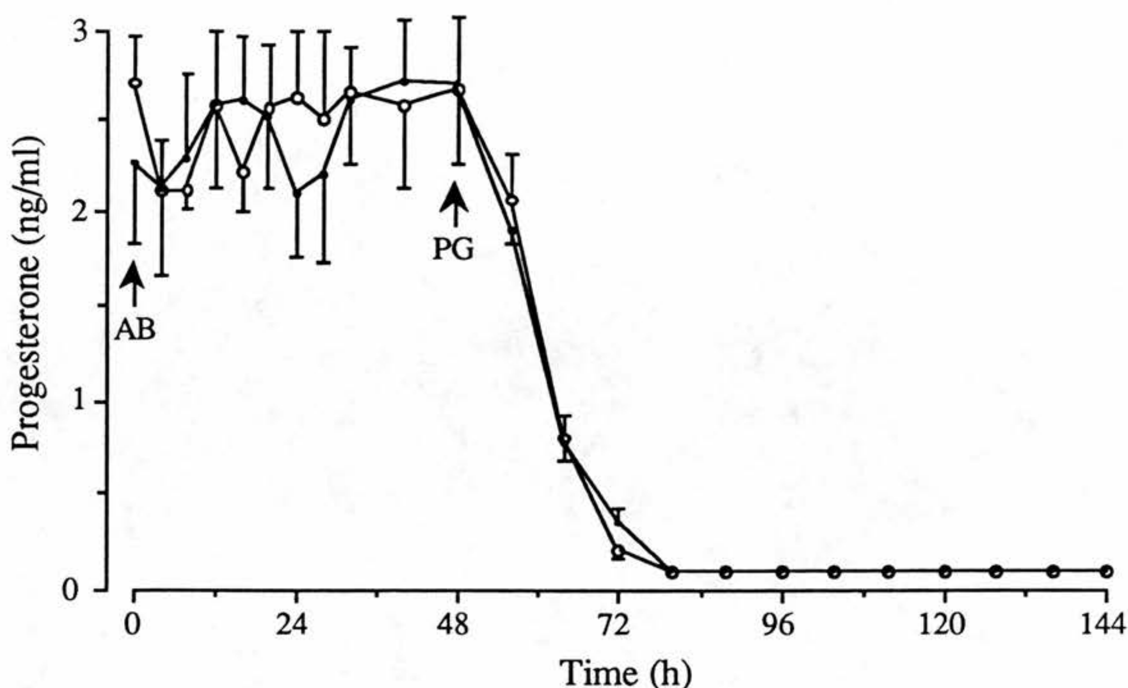

Fig. 6.5
Mean (± S.E.M.) concentration of progesterone in the plasma of ewes injected at time 0 with a single i.v. bolus of either (a) 10 ml nornal sheep plasma (NSP) or (b) 10 ml inhibin antibody (AB) before and after injection of cloprostenol (PG) at 48 h. (n = 5)

in both the control and the immunised group (54.4±3.9h and 54.4±1.6h respectively), as was the magnitude of the surge (86.2±18.7 ng/ml in the control group and 85.5±24.4 ng/ml in the immunised group).

In the control group the mean concentration of FSH in peripheral plasma was unaffected by injection of normal sheep plasma (Fig. 6.7.), concentration ranging from 1.65±0.02 ng/ml before treatment to 1.61±0.02 ng/ml during the 32h period following treatment. In the immunised group, in contrast to the control group, there was a significant ($p < 0.001$) response in the concentration of FSH in plasma following injection of antiserum (Fig. 6.7.). The concentration of FSH rose from a mean 1.42±0.06 ng/ml before treatment to a maximum of 2.58±0.23 ng/ml by 5.6±0.9h, this rise starting 3.8±0.7h following immunization. This maximum response lasted for 9.0±1.1h, but it was 32.8±6.9h before the concentration of FSH had returned to pre-treatment levels. As a result of this response, despite the initially lower baseline concentration of FSH in the immunised group compared to the control group (1.42±0.06 compared with 1.65±0.02 ng/ml), the concentration of FSH was significantly ($p < 0.05$) higher in the immunised group during the 32h period following antiserum injection (2.13±0.09 compared with 1.60±0.02 ng/ml). Following

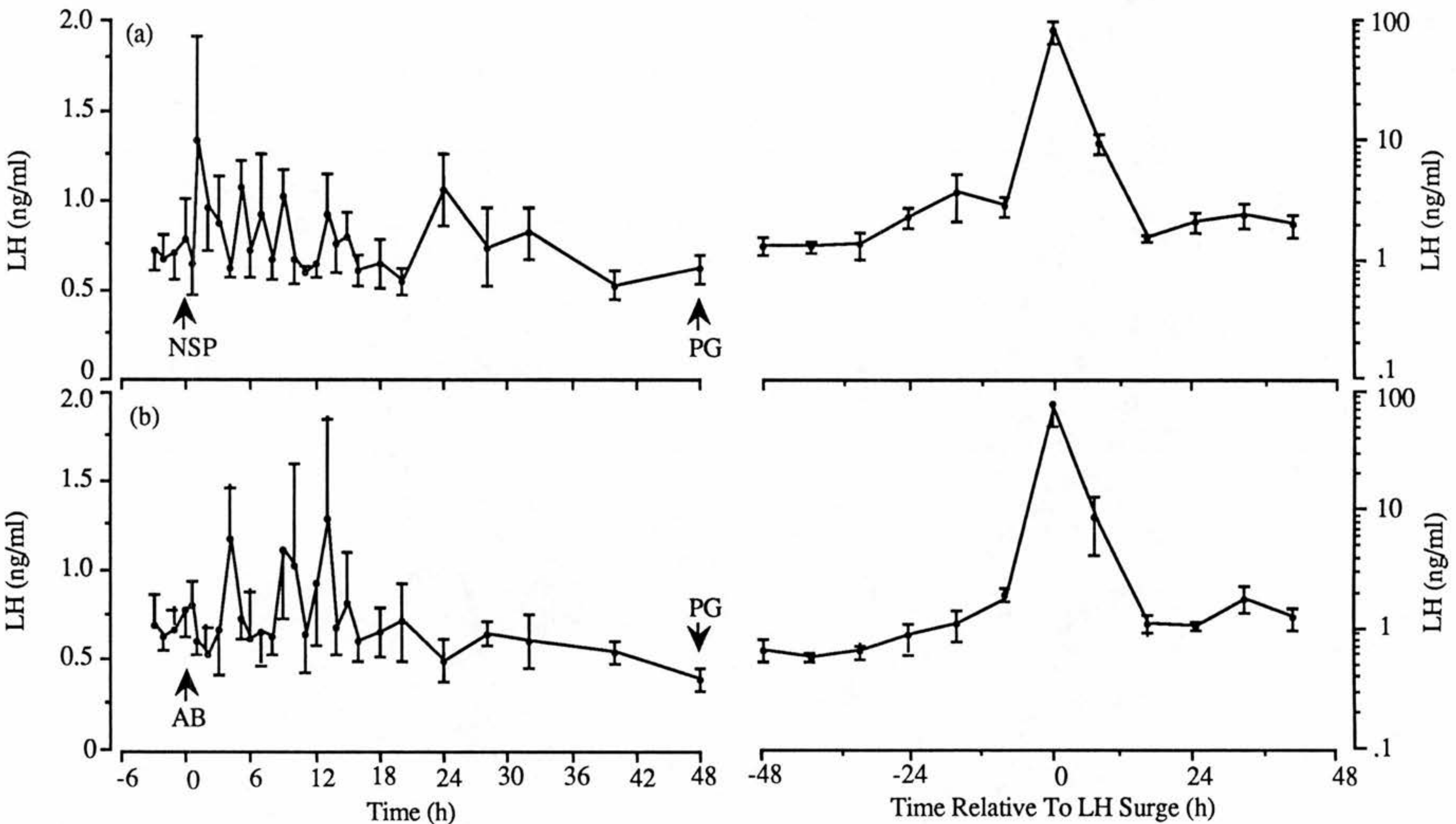

Fig. 6.6.
Mean (± S.E.M.) concentrations of LH in the plasma of ewes injected at time 0 with a single i.v. bolus of either (a) 10 ml nornal sheep plasma (NSP) or (b) 10 ml inhibin antibody (AB) and the subsequent LH surges following injection of cloprostenol (PG) at 48 h. (n = 5)

cloprostenol-induced luteal regression both groups showed normal FSH responses, exhibiting a primary surge coincident with that of LH and a second surge 16-24h later.

At laparoscopy there were three single and two double ovulations in the control group, and one single and four double ovulations in the immunised group. However, in the immunised animal with a single corpus luteum half the left ovary was hidden by an adhesion.

6.4. Discussion

In this study we have demonstrated that injection of antiserum to inhibin, during the luteal phase of the sheep oestrous cycle, results in a marked rise in the plasma concentration of FSH thus providing strong evidence that inhibin is involved in the negative feedback loop, from the ovary to the pituitary, regulating the production of FSH at this time.

As the injection of inhibin antiserum did not result in any effect on peripheral plasma concentrations of LH, this study supports the definition of inhibin as a selective suppressor of FSH. Following immunization a marked rise in the plasma concentration of FSH was observed in all animals injected with the inhibin antiserum. While the inhibin binding capacity in the plasma of immunised animals had reached a peak by 1h, the concentration of FSH did not start to rise until 3.8±0.7h after immunization. This delay in the response of FSH is similar in length to the delay in the response of FSH of 4-5h seen following unilateral ovariectomy (Findlay & Cumming, 1977; Campbell, 1988). A similar delay (3 - 4h) is also seen between injecting bovine follicular fluid and the onset of the resultant decline in FSH (McNeilly, 1985) indicating that a lag exists between the pituitary registering changes in plasma concentration of inhibin and its being able to respond with a change in output of FSH. The magnitude and duration of the rise in concentration of FSH following immunization is also similar to the response following unilateral ovariectomy (Findlay & Cumming, 1977; Campbell, 1988), a process involving the abrupt removal of half the ovarian factors regulating the pituitary release of FSH. As passive immunization against inhibin gave a similar response to unilateral ovariectomy, it would appear that inhibin is making a considerable contribution to the control of pituitary production of FSH during the luteal phase of the oestrous cycle.

During the period following the rise in FSH, when concentrations were starting to decline, there was little change in the inhibin binding capacity in the plasma of immunised ewes, indicating that the fall in the concentration of FSH was the result of

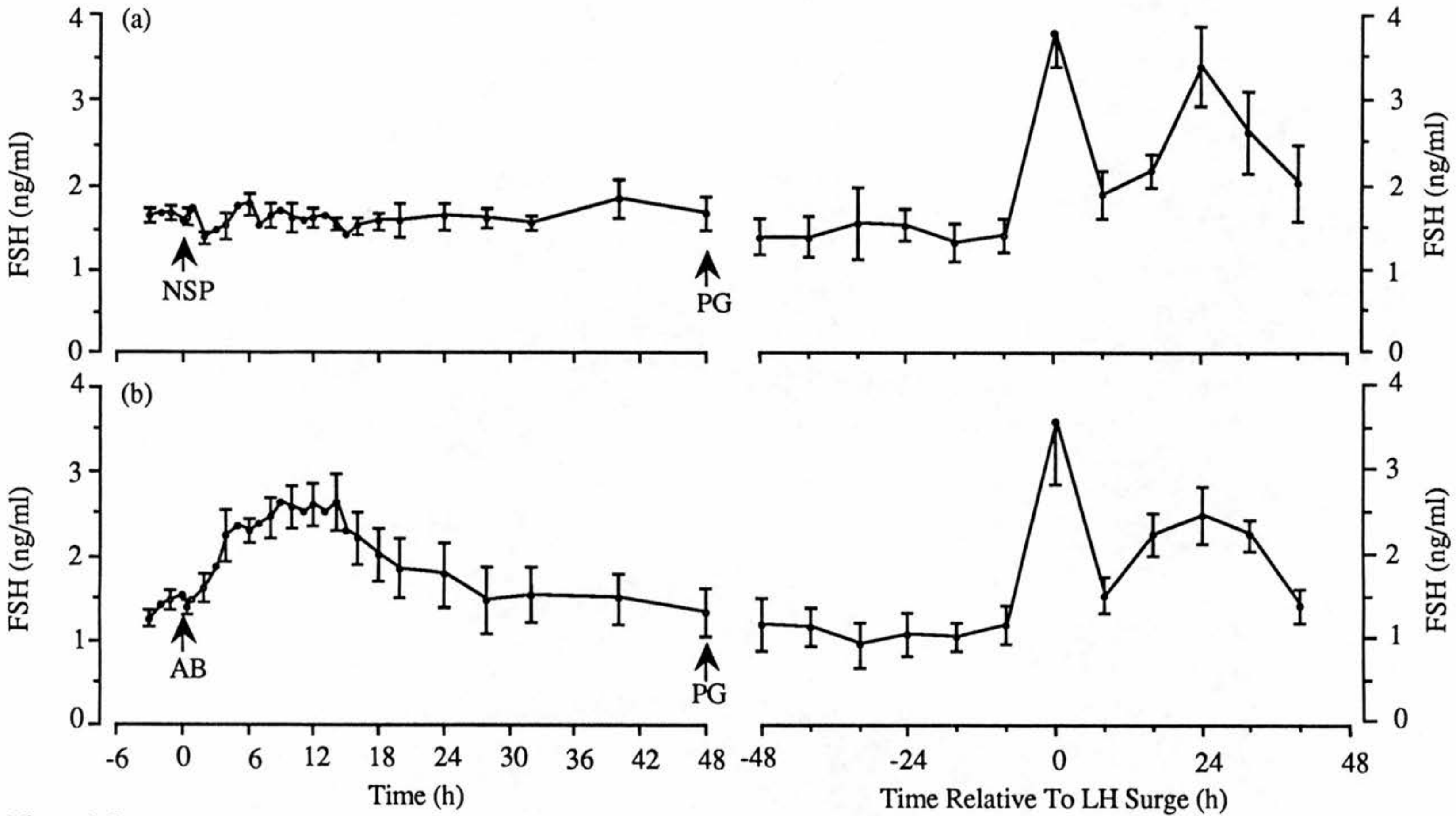

Fig. 6.7.
Mean (± S.E.M.) concentrations of FSH in the plasma of ewes injected at time 0 with a single i.v. bolus of either (a) 10 ml nornal sheep plasma (NSP) or (b) 10 ml inhibin antibody (AB) before and after injection of cloprostenol (PG) at 48 h. (n = 5)

some form of physiological compensation and not due to clearance of the antibody. The most likely mechanism by which the animals were compensating for the rise in FSH would be a rise in the ovarian secretion rates of inhibin and oestradiol.

In other studies in which inhibin antibodies have been successfully used to neutralise the effects of inhibin in suppressing the production of FSH by pituitary cells *in vitro* (Channing et al., 1982; McLachlan et al., 1986; van Dijk et al., 1986), antisera were raised to partially purified preparations of follicular fluid. This raises the possibility that in these studies, as well as antibodies against inhibin, antibodies against other follicular fluid products with inhibin-like activity might also have been present. In this study the antiserum was raised to a synthetic peptide fragment of inhibin, thus removing the possibility of the presence of antibodies against other follicular fluid products with inhibin-like effects. As such an antiserum was shown to completely neutralise the FSH suppressing effects of steroid-stripped oFF it appears that other than oestradiol, inhibin is the sole FSH suppressing substance present in the follicular fluid of sheep.

Studies involving active immunization against inhibin have all resulted in an increase in ovulation rate (Henderson et al., 1984; Cummins et al., 1986; Forage et al., 1987; Findlay et al., 1989; Wrathall et al., 1989). As inhibin suppresses the production of FSH it would seem likely that this increase in ovulation rate would be the result of neutralisation of inhibin resulting in an increase in the plasma concentration of FSH, which would in turn stimulate an increase in ovulation rate. However, these studies have reported small, inconsistent rises in the plasma concentration of FSH, or even no rise at all. In this acute, passive immunization study a highly significant rise in FSH was observed in all immunised animals. Within 48h, however, this rise had been compensated for in all animals, and no further differences in the levels of FSH were observed between control and immunised animals. As it appears that compensatory mechanisms can reverse the rise in FSH seen following passive immunization against inhibin, it may be that active immunization is not of a sufficiently acute nature to detect reliably the changes that are occurrring in plasma concentrations of FSH. This leads to the suggestion that following active immunization there may be a certain critical period during which such compensation is not possible and FSH levels become elevated, and that more extensive blood sampling is required to demonstrate the effects of active immunization on FSH. It may, however, be possible that only small rises in FSH are necessary to mediate the effects of inhibin immunization on ovulation rate. A further possibility may be that inhibin has an intra-ovarian role in the regulation of ovulation, and that neutralisation of inhibin by immunization can result in a rise in ovulation rate without the need for elevated plasma concentrations of FSH.

The results of the laparoscopy indicate that the immunization against inhibin may have resulted in an increase in ovulation rate, though numbers of animals and the degree of increase are too small to allow any firm conclusions to be drawn. The increase observed in this study (1.4 to 1.8) was, however, of a similar size to that seen in some active immunization studies (Henderson et al., 1984; Cummins et al., 1986), and supports a hypothesis that immunization against inhibin is a means of producing controlled increases in ovulation rate in sheep.

From the results of this study we conclude that passive immunization against inhibin during the luteal phase of the ovine oestrous cycle results in a marked rise in plasma concentration of FSH. While this effect can be compensated for, presumably by increased ovarian output of oestradiol, this study still provides very strong evidence that inhibin is an important factor in the regulation of FSH production by the pituitary gland at this time.

Chapter 7

Passive Immunisation against Inhibin and Oestradiol during the Follicular Phase of the Oestrous Cycle

7.1. Introduction

In the sheep it is well established that both oestradiol and inhibin can suppress the production of follicle stimulating hormone (FSH) (Findlay & Clarke, 1987). However, when doses of follicular fluid containing quantities of inhibin estimated as equivalent to normal ovarian output are administered to ewes only transient falls in the concentration of FSH are produced (Martin et al., 1987), while in the experiment described in Chapter 6 passive immunisation against inhibin produced only a short lived rise in the concentration of FSH. Oestradiol alone is unable to restore fully the normal concentration of FSH when administered to acutely ovariectomized ewes (Goodman et al., 1981; Martin et al., 1988) while together, inhibin and oestradiol can maintain normal FSH concentrations (Martin et al., 1988). It would appear, therefore, that these two hormones act together in the regulation of FSH production, though their relative importance still remains unclear.

Antisera raised to inhibin partially purified from bovine follicular fluid have been shown to neutralise the FSH-suppressive activity of inhibin in follicular fluid *in vitro* (van Dijk et al., 1986) but while active immunisation of ewes against inhibin from various sources all result in an increase in ovulation rate, such immunisations have not produced consistent increases in the concentration of FSH (Henderson et al., 1984; Cummins et al., 1986; Forage et al., 1987; Findlay et al., 1989; Wrathall et al., 1989). Active immunisation against oestradiol-17β has been shown to produce more consistent rises in FSH concentration, as well as increases in the concentration of LH (Pant et al., 1978; Rawlings et al., 1978; Martensz et al., 1979; Scaramuzzi et al., 1980) but such immunisations prevent ovulation by blocking the oestradiol-induced LH surge. In Chapter 6 neutralisation of endogenous inhibin by passive immunisation was shown to cause an increase in the plasma concentration of FSH, an effect that is also seen in rats (Rivier et al., 1986; Culler & Negro-Vilar, 1988). Passive immunisation with antibodies against oestradiol blocks the ability of oestradiol benzoate to induce oestrus in ovariectomized ewes (Scaramuzzi, 1975) and prevents ovulation in normal ewes when administered from day 13 to day 17 of the cycle (Fairclough et al., 1976). However, when a single immunisation was given in the luteal phase Pathiraja et al.

(1984) showed a transient rise in the concentration of FSH, together with an associated rise in ovulation rate.

In this study we have passively immunised ewes against inhibin and oestradiol, both separately and in combination, as a means of investigating the importance of these two hormones in the control of FSH production during the follicular phase of the sheep oestrous cycle.

7.2. Materials and Methods

7.2.1. Inhibin and Oestradiol Antibodies

The antibody to inhibin used in this study was raised in an ovariectomised ewe to a synthetic peptide of the 1 - 26 amino acid sequence of the N - terminus of the α chain of 32 kDa porcine inhibin and an appropriate dose of the antiserum was determined in the study described in Chapter 6. The oestradiol antibody was raised in an ovariectomised ewe to an oestradiol - rabbit serum albumin conjugate, and an appropriate dose was then determined in a pilot study similar to that used for the inhibin antibody.

7.2.2. Pilot Dose Study

A dose trial was performed during the breeding season in January on six mature Blackface ewes in order to determine a suitable dose of oestradiol antibody to use in the main experiment. Oestrous cycles were synchronised and animals allocated to three groups of two animals with similar mean group weights of approximately 50 kg. On day 10 of the luteal phase animals were given an i.v. bolus injection of either 1 ml, 5 ml, or 10 ml oestradiol antibody . Hourly venous blood samples were collected by vacutainer for 3 h before and 2 hourly sample for 48 h after injection of antibody. Samples were then assayed for FSH.

7.2.3. Immunisation Experiment

The immunisation experiment was carried out during the breeding season, in February, on 20 mature Scottish Blackface ewes. Oestrous cycles were synchronized and the animals were then allocated to four groups of five animals with similar mean

group weights (control, 57±2kg (±S.E.M.); inhibin immunisation, 55±4kg; oestradiol immunisation, 55±2kg; combined immunisation, 56±4kg) and their jugular veins cannulated using plastic cannulae with three-way taps. On day 10 of the luteal phase luteal regression was induced by a further i.m. injection of Cloprostenol (100 µg) and 24h later animals were injected with a single i.v. bolus injection of one of four treatments:

A. Control - 10ml normal sheep plasma (n=5)
B. Inhibin Immunised - 10ml inhibin antibody (n=5)
C. Oestradiol Immunised - 10ml oestradiol antibody (n=5)
D. Combined - 10ml inhibin antibody and 10ml oestradiol antibody (n=5).

Samples of jugular venous blood (5ml) were collected every 2h from 8h before until 24h after the induction of luteal regression (at time 0h). Following administration of antibody at 24h samples were collected at decreasing time intervals (24-48h, 1 hourly; 48-72h, 2 hourly; 72-168h, 4 hourly; 168-228h, 12 hourly) until the end of the experiment. Blood samples were then assayed by radioimmunoassay for FSH, luteinizing hormone (LH), and progesterone and the inhibin and oestradiol binding capacities in the plasma of immunised ewes was determined.

7.2.4. Analysis of Data

The rise in FSH in response to immunisation was defined to have commenced at the first point on a sequential FSH rise with an increase in concentration over the previous sample in excess of the sum of the assay error, and to have ended at the point where the concentration was no longer greater than pretreatment level by more than the assay error. The assay error was estimated from the coefficient of variation determined in a plasma pool containing approximately the same concentration of FSH, and was defined as 2x the standard deviation at that concentration.

Hormone concentrations and binding capacities were compared both within and between groups by analysis of variance on repeated samples and LH surge characteristics and the components of FSH responses compared between groups by a Student's unpaired t-test.

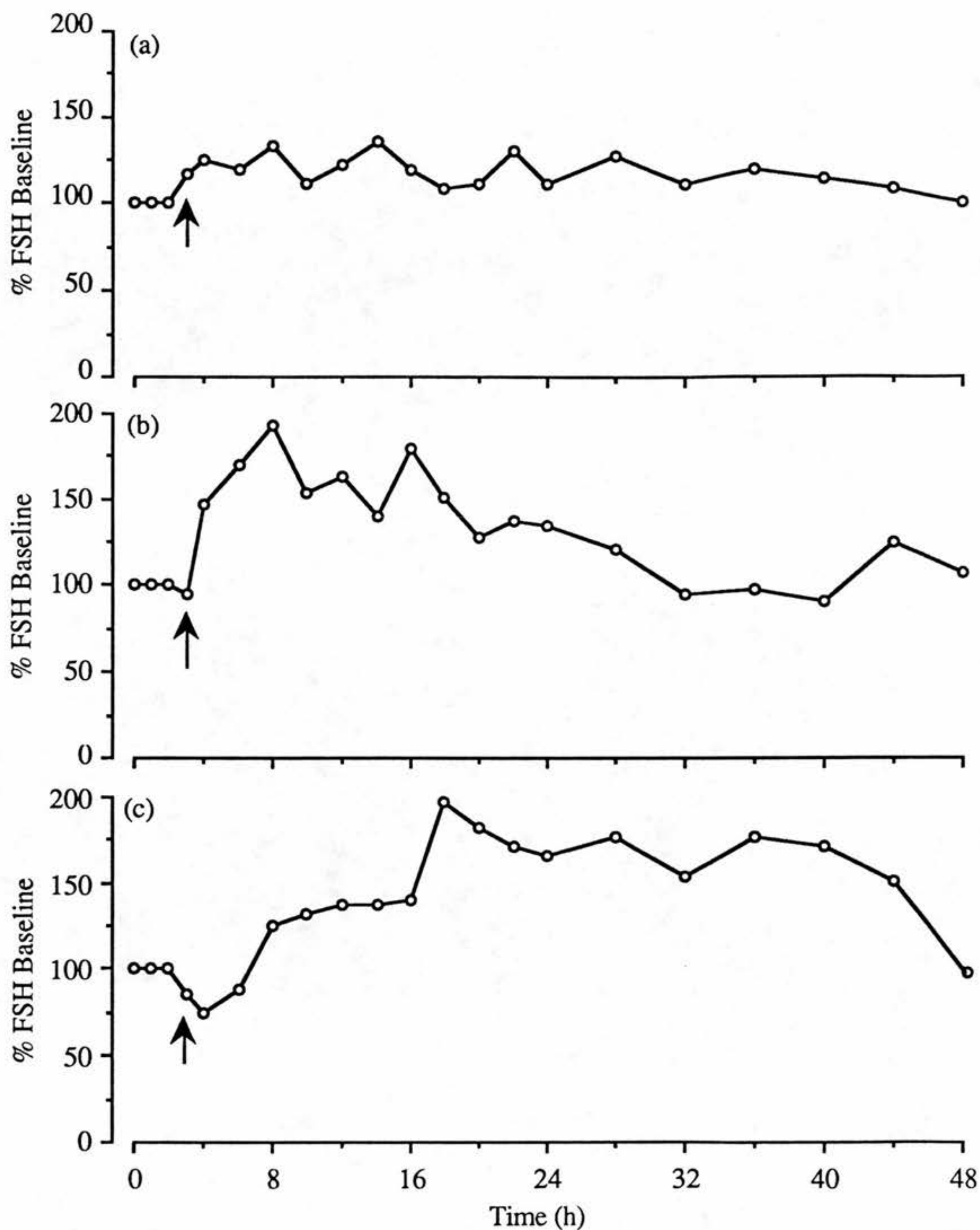

Fig. 7.1.
Plasma concentration of FSH as a percentage of the pretreatment baseline concentration resulting from the injection at time 0 of an i.v. bolus of (a) 1 ml, (b) 5 ml or (c) 10 ml of oestradiol antibody. (Values are means of duplicate determinations).

7.3. Results

7.3.1. Pilot Dose Study

Both the 5 ml and 10 ml groups showed a marked rise in plasma FSH concentration following antibody injection , while the 1 ml group showed a smaller rise in FSH concentration (Fig 7.1). In the 1ml group FSH concentration rose from a mean of 1.80 ng/ml before treatment to a maximum of 2.37 ng/ml (mean 135%; range 120 to 151%), in the 5ml group this rise was from 2.13 to 3.55 ng/ml (mean 160%; range 134 to 187%) and in the 10ml group 1.75 to 3.12 ng/ml (mean 180%; range 147 to 213%). Due to the small group size, and the variable nature of the responses it is not possible to draw firm conclusions, but it would appear that while the 5 ml and 10 ml doses gave a similar magnitude of response the 10 ml antibody injection resulted in a more sustained response, and so a dose of 10 ml was decided upon.

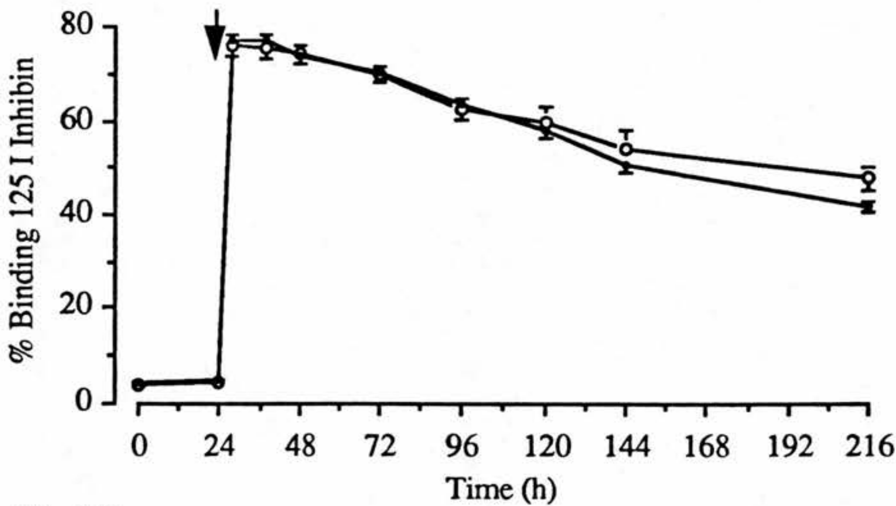

Fig. 7.2.
(a) mean (±S.E.M.) inhibin binding capacity (at a dilution of 1:15) in the plasma of ewes injected at time 24 h with a 10 ml i.v. bolus of either inhibin antibody alone (o; n = 5) or in combination with a 10 ml i.v. bolus injection of oestradiol antibody (•; n= 5)

7.3.2. Plasma - Binding Capacity

Following immunisation there was an increase in the ability of plasma to bind inhibin and/or oestradiol in all the immunised animals (Fig. 7.2 & 7.3). In the animals immunised against inhbin (Fig. 7.2), by 2h following immunisation, plasma inhibin-binding capacity, at a dilution of 1:15, was 76.3±2.3 % (± S.E.M.) in the inhibin immunised group and 77.0±1.3 % in the combined immunisation group. The binding capacity then showed a similar steady decline ($p < 0.001$) over the course of the experiment in both groups, falling to 59.9±3.3 and 58.1±1.7 % by 120h and to 47.7±2.5 and 41.8±1.0 % by 216h in the inhibin immunised and combined immunisation groups respectively. In the oestradiol-immunised animals, by 2h following antibody injection, plasma oestradiol-binding capacity, at a dilution of 1:400, was 68.7±1.3 % in the oestradiol-immunised group and 67.0±0.5 % in the combined immunisation group. Like the inhibin-binding capacity, the oestradiol-binding capacity then showed a steady decline ($p < 0.001$) which did not differ significantly between the two groups, falling to 48.4±2.6 and 45.2±1.6 % by 120h and to 37.6±4.4 and 35.9±3.5 % by 216h in the oestradiol-immunised and combined immunisation groups respectively.

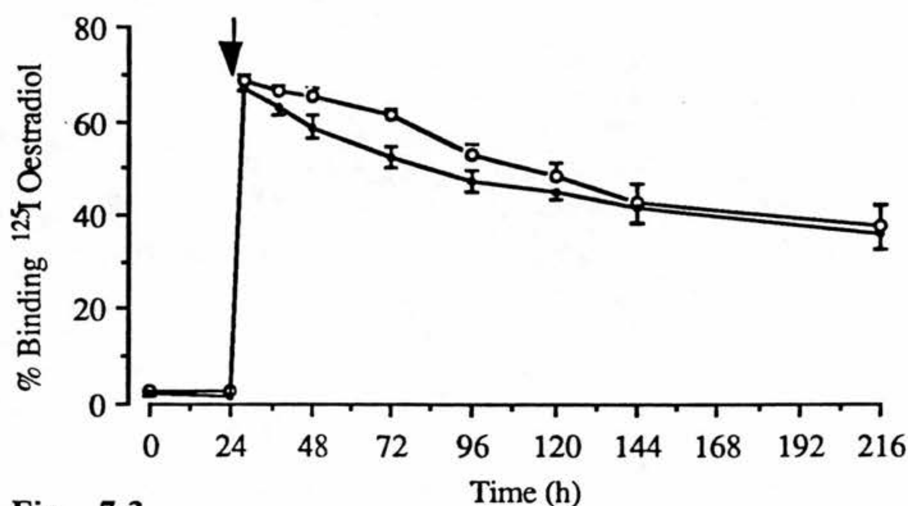

Fig. 7.3.
(a) mean (±S.E.M.) oestradiol binding capacity (at a dilution of 1:15) in the plasma of ewes injected at time 24 h with a 10 ml i.v. bolus of either oestradiol antibody alone (o; n = 5) or in combination with a 10 ml i.v. bolus injection of inhibin antibody (•; n= 5)

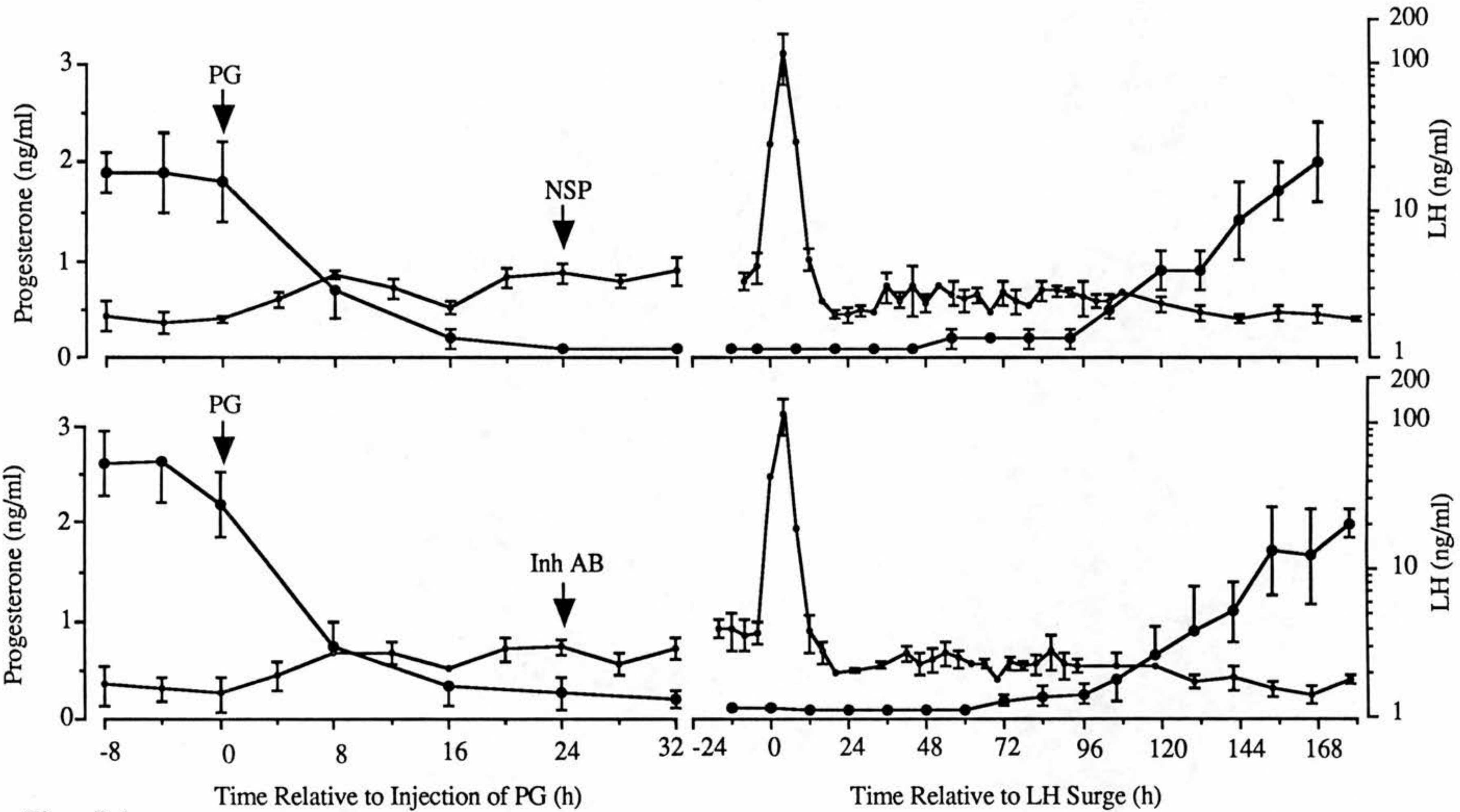

Fig. 7.4.
Mean (± S.E.M.) plasma concentration of LH (•) and progesterone (●) in the plasma of ewes injected at time 0 with 100 µg cloprostenol (PG) and at time 24 h with a 10 ml i.v. bolus injection of either (a) normal sheep plasma (NSP; n = 5) or (b) inhibin antibody (Inh AB; n = 5).

7.3.3. Progesterone

Following Cloprostenol-induced luteal regression the concentration of progesterone had fallen significantly ($p < 0.001$) by 16h in all four groups (Figs 7.4 and 7.5), and by 32h the concentration of progesterone was less than 0.1 ng/ml in all groups. In both the control and inhibin immunised groups the concentration of progesterone rose significantly ($p < 0.001$) following the LH surge (Fig.7.2), this rise starting on day 4 following the surge and reaching 1.82±0.35 ng/ml in the control group and 2.13±0.63 ng/ml in the inhibin-immunised group by day 7. The timing and magnitude of this rise was not different between the two groups. In the oestradiol-immunised and combined immunisation groups the concentration of progesterone showed no rise at this time.

7.3.4. Luteinizing Hormone

During the 12h period following Cloprostenol - induced luteal regression at time 0h there was a significant ($p < 0.01$) rise in the concentration of LH in all four groups (Figs 7.4 and 7.5). Following injection of normal sheep plasma (control) or inhibin antibody (inhibin immunised) there was no significant change in the plasma concentration of LH during the period before the onset of the LH surges (Fig 7.4). In these two groups luteal regression was followed by an LH surge in all animals, the timing (control, 45.0±4.6h; inhibin-immunised, 56.2±8.2h), duration (control, 8.4±0.8h; inhibin-immunised, 7.4±1.1h) and magnitude (control, 149.7±25.2 ng/ml; inhibin-immunised, 117.2±24.5 ng/ml) of these surges not differing significantly between the two groups. However, in animals immunised against oestradiol either alone or in combination with inhibin an LH surge did not occur (Fig. 7.5). In both these groups there was a significant increase in plasma concentration of LH with time during the 96h period following antibody administration (oestradiol immunised $p < 0.05$; combined immunisation $p < 0.01$), this response not differing significantly between the two groups. The mean concentrations of LH over this period compared with the 12h period before administration of antibody were 3.4±0.2 compared with 2.8±0.3 ngm/l in the oestradiol-immunised group (26.3±9.9% rise) and 3.3±0.4 compared with 2.6±0.2 ng/ml in the combined immunisation group (31.1±9.1% rise).

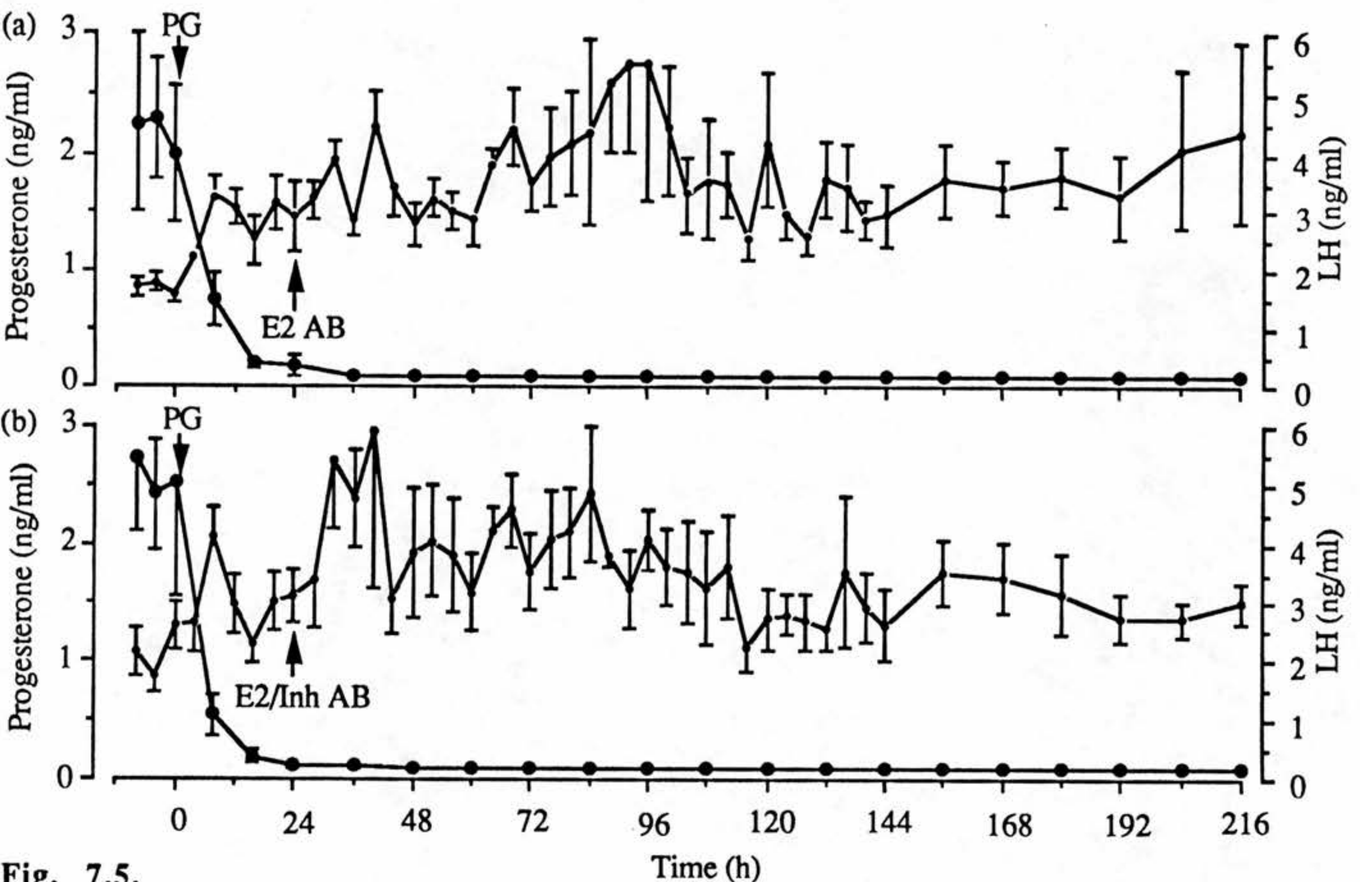

Fig. 7.5.
Mean (± S.E.M.) plasma concentration of LH(•) and progesterone (●) in the plasma of ewes injected at time 0 with 100 µg cloprostenol (PG) and at time 24 h with a 10 ml i.v. bolus injection of oestradiol antibody either (a) alone (E2 AB; n = 5) or (b) in combination with a 10 ml i.v. bolus of inhibin antibody (E2/Inh AB; n = 5).

7.3.5. Follicle Stimulating Hormone

Following the induction of luteal regression at time 0h with Cloprostenol there was a significant ($p < 0.001$) decline in the plasma concentration of FSH in all four groups (Fig. 7.6). During the 6 h period before antibody administration all four groups showed no significant changes in the concentration of FSH. During the 14 h period following normal sheep plasma (NSP) injection there was no significant change in the mean concentration of FSH compared with the mean of the previous 6 h period (1.6±0.1 compared with 1.6±0.1 ng/ml; Fig. 8.4). Following the administration of antibodies there was a significant ($p < 0.001$) increase in the concentration of FSH during this period in both the inhibin- and oestradiol-immunised groups. Following the combined immunisation there was also a significant ($p < 0.001$) increase in the mean concentration of FSH, which was significantly greater than the rise seen following the two individual immunisations ($p < 0.01$). Due to differences in the duration of FSH responses between animals, and the lack of LH surge - associated rises in FSH in the two oestradiol immunised groups, when examining entire responses it is more appropriate to look at individual representative profiles than at mean graphs. Such profiles are shown in Fig. 7.7, while the characteristics of the responses in FSH concentration following immunisation are shown in Table 7.1. In all the control animals there was no change in the concentration of FSH following NSP injection until the first FSH surge. In four of the five inhibin immunised animals the response in FSH concentration started 4.8±0.6h following antibody injection and finished 17.0±0.5h later while in the fifth animal the FSH response started 5h following antibody injection but the FSH surge occurred before the end of the FSH response and so this animal was excluded from further analysis. The time to the onset of the rise in FSH did not differ significantly between the three immunisation groups, while the duration of the response was significantly ($p < 0.05$) greater in the oestradiol immunised and combined immunisation groups than in the inhibin immunised group. When the mean concentrations of FSH during the responses to immunisation were compared with the mean concentrations of FSH during the 6h period before antibody administration in each group the inhibin and oestradiol immunisations were found to give rises of 42.0±6.1 and 42.6±3.5% respectively, while the combined immunisation resulted in a significantly ($p < 0.01$) greater rise of 95.2±16.6%.

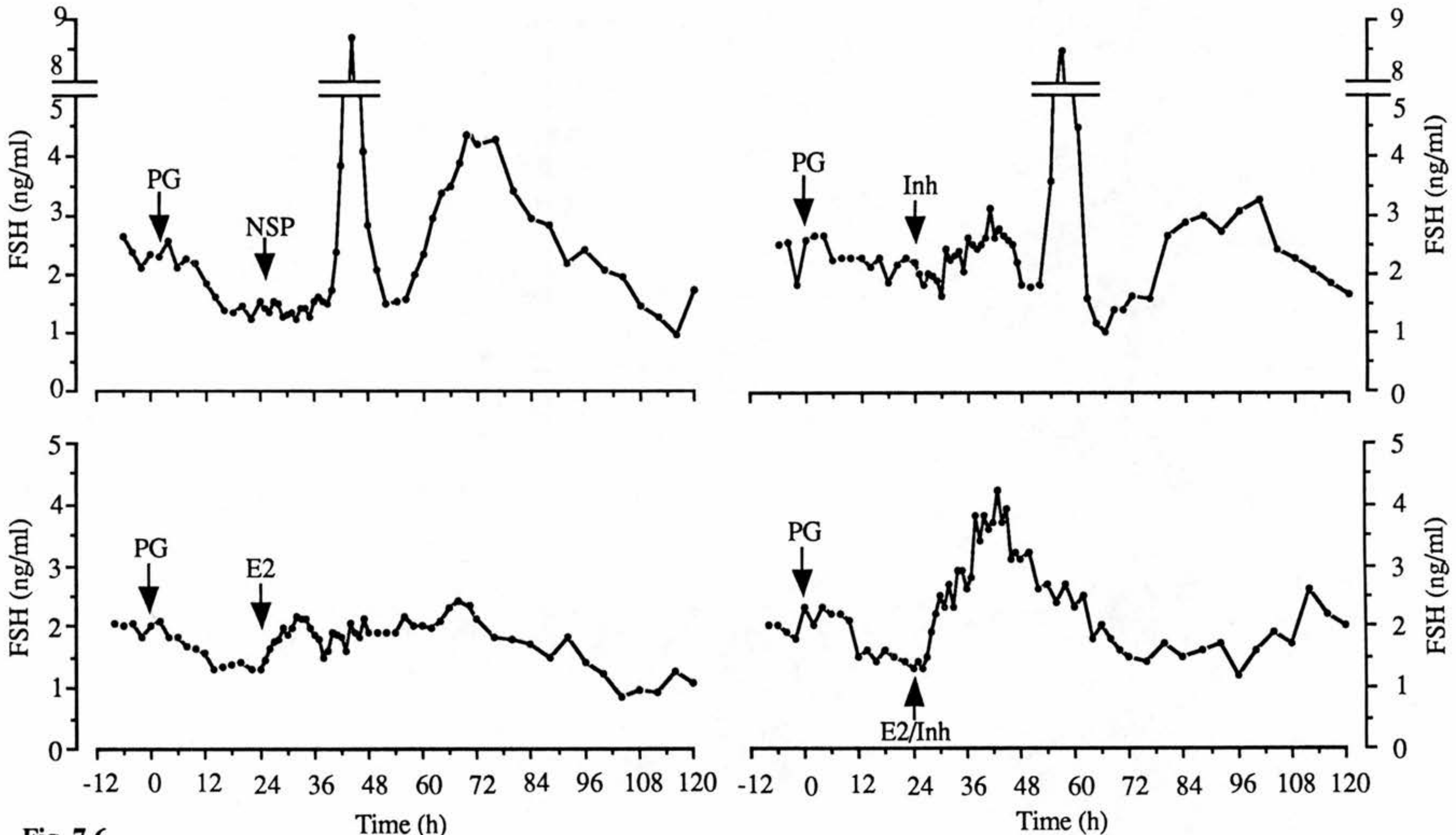

Fig. 7.6.
Individual representative FSH profiles from groups of ewes injected at time 0 with 100 µg cloprostenol and at time 24 h with an i.v. bolus of either; (a) 10 ml normal sheep plasma (NSP), (b) 10 ml inhibin antibody (Inh), (c) 10 ml oestradiol antibody (E2) or (d) 10 ml inhibin antibody and 10 ml oestradiol antibody in combination (E2/Inh)

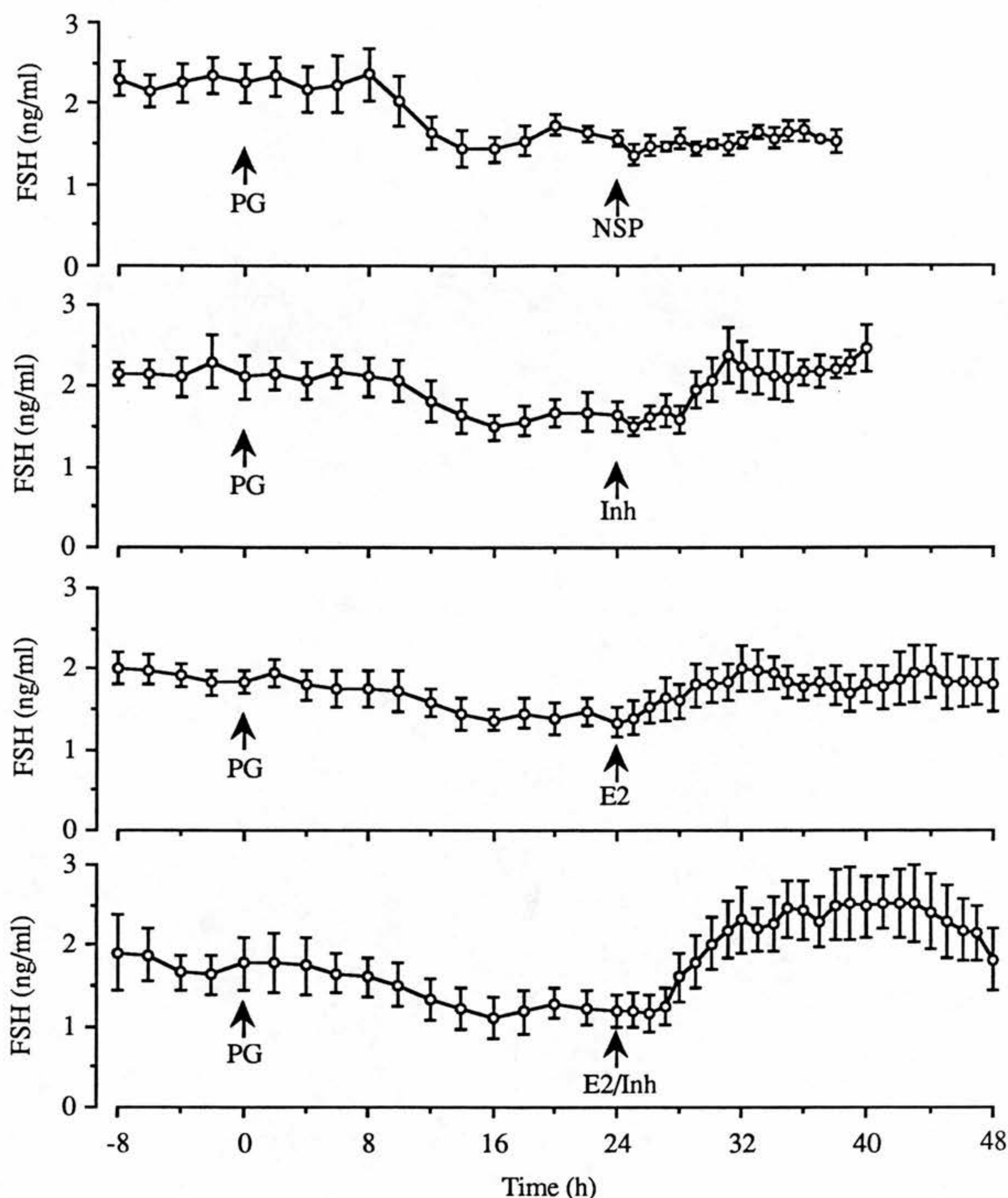

Fig. 7.7.
Mean (± S.E.M.) plasma concentration of FSH following injection at time 0 with 100 μg cloprostenol and at time 24 h with an i.v. bolus of either; (a) 10 ml normal sheep plasma (NSP; n = 5), (b) 10 ml inhibin antibody (Inh; n = 5), (c) 10 ml oestradiol antibody (E2; n = 5) or (d) 10 ml inhibin antibody and 10 ml oestradiol antibody in combination (E2/Inh; n = 5). In the control and inhibin immunised animals the graphs cease at the time when the first animal started an LH surge.

Table 7.1 Characteristics of the FSH responses in the three immunised groups

Group	Time to Response	% Rise	Duration
Inhibin	4.8 ± 0.6 h	42.0 ± 6.1	17.0 ± 0.5 h
Oestradiol	3.8 ± 0.5 h	42.6 ± 3.5	43.6 ± 12.8 h
Combined	4.4 ± 0.6 h	95.2 ± 16.6	40.6 ± 11.7 h

7.4. Discussion

In this study we have shown that the injection of antisera to both oestradiol and inhibin, during the follicular phase of the oestrous cycle, results in a rise in the peripheral plasma concentration of FSH. This, together with the fact that the injection of both oestradiol and inhibin antisera simultaneously resulted in a larger increase in the concentration of FSH demonstrates that both oestradiol and inhibin are involved in the control of FSH production at this stage of the cycle.

In all control and inhibin immunised animals the LH surge was followed by a rise in progesterone to normal luteal phase concentrations, indicating ovulation and formation of the corpus luteum had occurred normally, and had not been affected by the inhibin antibody. In contrast, no animals showed such a rise in progesterone in the two groups immunised against oestradiol, indicating that ovulation had been blocked, due to the failure of the oestradiol-induced preovulatory LH surge. Earlier studies involving passive immunisation have shown a block of oestrus and ovulation (Scaramuzzi, 1975, Fairclough et al., 1976), but in the study of Pathiraja et al. (1984) ovulation was not blocked, as a single antiserum injection was given earlier in the luteal phase. In the present study, as in the experiment described in Chapter 6, the administration of inhibin antibodies had no effect on either the concentration of LH in the peripheral plasma or the characteristics of the LH surge, this finding adding further weight to the hypothesis that inhibin has no role in the control of LH production.

Following luteal regression in the sheep, there is a marked fall in the concentration of FSH, while at the same time oestradiol, the classical hormonal regulator of FSH, shows a marked increase (Baird & McNeilly, 1981). During this period McNeilly et al., (1989) found little change in immunoactive inhibin concentration, while Tsonis et al. (1988b) found a fall in inhibin bioactivity. However, immunoactive inhibin concentration has also been found to exhibit moderate increases at this time (Findlay et al., 1990; Campbell et al., 1990a). From these reports it would

seem unlikely that inhibin has a major role in regulating the fall in FSH concentration following luteal regression, and that oestradiol is the major factor involved in this process. However, in this study the injection of inhibin antibodies 24h after luteal regression resulted in a significant ($p < 0.001$) rise in the concentration of FSH. This supports a hypothesis that, while changes in oestradiol secretion are responsible for the change in the pattern of FSH concentration, the absolute concentration of FSH is under the joint control of oestradiol and inhibin. Thus oestradiol with its fast metabolic clearance rate (1600ml/min; Challis et al., 1970) may modulate the change in the concentration of FSH while combining with inhibin, with its much slower clearance rate (21ml/min, see Chapter 3), to regulate the actual level to which FSH concentration is suppressed. As a result of the oestradiol immunisation there was a moderate increase in FSH concentration (43%) to a level not significantly different to that seen before luteal regression, the magnitude of this rise being similar to that produced by the inhibin immunisation (42%). The combined immunisation with oestradiol and inhibin resulted in a rise in FSH concentration approximately double that seen following the individual immunisations (95%), providing further evidence that oestradiol and inhibin act in combination to regulate the concentration of FSH.

The increase in FSH concentration following inhibin immunisation lasted approximately 17h while the response to oestradiol and combined immunisations lasted significantly longer (44 and 41h respectively). During these periods there was only a small decline in the respective binding capacities of plasma, and so the compensatory fall in the concentration of FSH was probably not due to clearance of antibody. It would seem likely that following the rise in FSH concentration, after inhibin immunisation, the compensatory fall in concentration of FSH may be due to an increase in the secretion of oestradiol by the ovary, while following oestradiol immunisation there may be an increase in inhibin output. A similar increase in the secretion of inhibin occurs following active immunisation of ewes against androstenedione which results in multiple follicular development but near normal levels of FSH (Campbell et al., 1988). The rise in the concentration of FSH following injection of inhibin antibodies during the follicular phase was smaller and more short lived than that obsereved following the luteal phase inhibin immunisation described in Chapter 6, probably as a result of the increased availability of oestradiol, secreted by the large preovulatory follicles during the follicular phase (Baird & McNeilly, 1981).

In this study we have shown that passive immunisation against oestradiol or inhibin during the follicular phase of the sheep oestrous cycle resulted in a rise in the plasma concentration of FSH. From this we conclude that as well as oestradiol, inhibin is also important in the regulation of FSH production at this time.

Chapter 8

Passive Immunisation against Inhibin and/or Oestradiol During the Luteal Phase, and Replacement of Inhibin and/or Oestradiol Following Acute Ovariectomy

8.1. Introduction

From the results of the studies described in Chapters 6 and 7 it seems clear that inhibin and oestradiol are acting together in the control of FSH secretion. This idea is supported by a number of studies including one by Martin at al. (1988) in which "physiological" doses of inhibin and oestradiol were each shown to partly negate the post - castration rise in FSH in ewes, while a combination of the two treatments completely prevented this rise. The idea that oestradiol is only partly responsibe for the control of FSH secretion is further supported by the fact that "physiological" doses of oestradiol and progesterone will only partly prevent the rise in FSH seen following ovariectomy, while maintaining LH concentration (Goodman et al., 1981). Compared to the control of FSH secretion, the control of LH secretion is more clearly understood and appears to be almost entirely under the joint control of oestradiol and progesterone during the breeding season (Martin, 1984). There are, however some conflicting reports on the nature of this control mechanism, and further investigation is required. For example Karsch et al. (1977) found that progesterone alone was able to prevent the post - castration rise in LH for up to 48h while Martin et al. (1988) found that progesterone was ineffective in preventing this rise.

In Chapter 7 immunisation against inhibin or oestradiol during the follicular phase of the cycle was shown to cause a rise in FSH concentration, while a combined immunisation against both hormones resulted in a much larger rise. In this experiment the approach of single immunisations against inhibin or oestradiol in conjunction with a combined immunisation against both hormones was repeated during the luteal phase to investigate the interaction between these two hormones at this time. The "classical" approach to investigating the importance of steroids in the control of gonadotrophin secretion has involved the use of steroid - releasing implants, while much of our knowledge of the role of inhibin in this process has come from experiments involving the injection of inhibin in follicular fluid from which steroids have been removed by charcoal treatment. In this study these two approaches were combined with passive immunisation in the same experiment in an attempt to achieve further clarification of the

interaction between steroid hormones and inhibin in the control of gonadotrophin secretion in the ewe.

8.2. Materials and Methods

8.2.1. Experimental Animals

The experiment was carried out during the breeding season, in January, on 43 mature Scottish Blackface ewes. Oestrous cycles were synchronized and the animals were then allocated to nine groups of four to five animals with similar mean group weights (58.1±0.3):

A. Ovariectomised Control (Ovx; n = 5)
B. Ovx + Progesterone Implants (P_4 Imp) (Ovx/P_4; n = 5)
C. Ovx + P_4 Imp + 0.8ml oFF/8h (Ovx/P_4/Inh; n = 5)
D. Ovx + P_4 Imp + 1cm E_2 Implant (Ovx/P_4/E_2; n = 4)
E. Ovx + P_4 Imp + 1cm E_2 Implant + 0.8ml oFF/8h (Ovx/P_4/Inh/E_2; n = 4)
F. Control (10ml normal sheep plasma; n = 5)
G. Inhibin Immunised (10ml inhibin antibody, Inh Imm; n = 5)
H. E_2 Immunised (10ml E_2 antibody, E_2 Imm; n = 5)
I. Combined Immunisation (10ml inhibin & 10ml E_2 antibody, E_2/Inh Imm; n = 5)

Animals in Groups A to E were ovariectomised on day 10 of the luteal phase and in Groups B to E animals were simultaneously treated with P_4 implants to maintain luteal phase progesterone levels. Animals in Group C were also injected at 8h intervals with 0.8 ml "steroid stripped" oFF to replace ovarian inhibin production while in Group D animals were treated with oestradiol implants to replace ovarian oestradiol secretion. In Group E animals were treated with both oestradiol implants and "steroid stripped" oFF injections to replace both ovarian inhibin and oestradiol production. Animals in Group F were injected with 10ml normal sheep plasma to act as a control group while animals in Groups G and H were injected with 10ml inhibin and oestradiol antibody respectively to immunoneutralise these two hormones individually. Finally animals in Group I were injected with 10ml of inhibin antibody and 10ml oestradiol antibody in order to neutralise the two hormones simultaneously. Jugular veins were cannulated using plastic cannulae with three-way taps on day 9 of the luteal phase of the oestrous cycle and blood samples were collected at 2h intervals from 10h before treatment on day 10 until 48 after treatment. Between 12 and 24h after treatment blood samples were

collected at 10min intervals to allow the pulsatile release of LH the be measured. After collection samples were frozen at - 20°C until assayed for FSH, LH and progesterone.

8.2.2. Ovariectomy

Anasthesia was induced using a mixture of two parts thiopentone (Intraval; RMB Animal Health Ltd, Dagenham. Essex, U.K.) and one part pentobarbitone sodium (Sagatal; May and Barker Ltd, Dagenham, Essex, U.K.), and if necessary further anaesthetic mixture was injected during ovariectomy via the jugular cannula. Ovaries were then removed through a mid - ventral incision. Within each group ovariectomies were performed within a one hour period, with the two hourly blood samples collected 1.5 - 2.5h before and 1.5 - 2.5h after removal of the ovaries.

8.2.3. Steroid and Inhibin Treatments

Progesterone and/or oestradiol was administered by means of hormone - releasing silastic implants which were inserted subcutaneously at the time of ovariectomy. The progesterone implants used in this study contained 375 mg (10%) progesterone in a silicone elastomer (Silestrus Implants; Cevar, Southampton, U.K.). Two such implants were inserted into each animal, a number which has been shown to generate luteal phase progesterone concentrations (Martin et al., 1988). The oestradiol implants used in this study were similar to those described by Goodman et al. (1981) and were made by filling lengths of Silastic tubing (i.d. 0.132" o.d. 0.183"; Dow Corning Silastic Medical Grade Elastomer; Hospital Management Supplies, Carnegie Rd, Glasgow, U.K.) with oestradiol 17β and sealing the ends with Silastic plugs and Silastic rubber adhesive. When incubated with agitation at 37°C, in phosphate buffered 0.9% saline containing 1% BSA, these implants released 2.1±0.1μg/24h. Prior to use, the implants were soaked at room temperature in distilled water over a 24h period to dissipate surface oestradiol contamination and prevent excessive release during the period immediately following insertion. Prior to insertion the implants were washed several times with sterile 0.9% saline. The implants used in this study were 1 cm in length, a size of implant that has been shown to generate luteal phase levels of oestradiol in the peripheral circulation (Martin et al., 1988). The inhibin treatment used in this study involved subcutaneous injections of ovine follicular fluid from a pool which had been charcoal - stripped and passed through C18 Sep-pak columns to remove steroids (see Section 2.7.). In the experiment in Chapter 5 1ml injections of this pool of oFF at 8h intervals was found to generate peripheral concentrations at the top of

the physiological range and so in this study injections of 0.8ml oFF at 8h intervals were used. The oFF had an inhibin bioactivity of 8.7kU/ml measured in the ovine pituitary cell inhibin bioassay of Tsonis et al. (1986) and so the dose used represented 7kU/8h.

8.2.4. Antibody Treatments

The antibody to inhibin used in this study was raised in an ovariectomised ewe to a synthetic peptide of the 1 - 26 amino acid sequence of the N - terminus of the α chain of 32 kDa porcine inhibin (see Section 2.6) and a dose of 10ml of the antiserum was found to give a maximum response in the study described in Chapter 6. The oestradiol antibody was raised in an ovariectomised ewe to an oestradiol - rabbit serum albumin conjugate (see Section 2.5), and a dose of 10ml shown to give a maximum response in a pilot study described in Chapter 7.

8.2.5. Statistical Analysis

Progesterone concentrations were analysed using analysis of variance on repeated samples and Duncan's multiple range test. Mean peripheral concentrations of FSH and LH over the experimental period were analysed using analysis of variance on data that had been converted to a percentage of the mean pretreatment concentration in order to compensate for differences in initial mean group concentrations. LH pulse characteristics were analysed using the Munro Pulse Analysis programme. The G parameters used (the number of standard deviation units by which a peak must exceed the baseline concentration at that time to be accepted as a pulse), G1 - G5, were 5, 3.5, 2.0, 1.5 and 1.0 standard deviation for pulses containing 1 - 5 samples above baseline concentration. Differences in pulse characteristics were then compared between groups using a student's unpaired t - test on data which had been log transformed due to large within group variation. In the immunised animals the rise in FSH in response to immunisation was defined to have commenced at the first point on a sequential FSH rise with an increase in concentration over the previous sample in excess of the sum of the assay error, and to have ended at the point where the concentration was no longer greater than pretreatment level by more than the assay error. The assay error was estimated from the coefficient of variation determined in a plasma pool containing approximately the same concentration of FSH, and was defined as 2x the standard deviation at that concentration. Characteristics of the FSH responses were compared using a Student's unpaired t -test.

8.3. Results

8.3.1. Plasma Hormone Binding Capacity

Following immunisation there was an increase ($p < 0.001$) in the ability of plasma to bind inhibin and/or oestradiol in all the immunised animals. In the animals immunised against inhibin, by 4h following immunisation, plasma inhibin-binding capacity, at a dilution of 1:15, was 71.4±2.3 % (± S.E.M.) in the inhibin immunised group (G) and 68.9±3.1% in the combined immunisation group (I) compared to a pre-immunisation non specific binding of <5% in both groups. The binding capacity then showed a similar steady decline ($p < 0.001$) over the course of the experiment in both groups, falling 64.3±2.9 and 62.7±3.4% by 48h in the inhibin immunised (G) and combined immunisation (I) groups respectively. In the oestradiol-immunised animals, by 2h following antibody injection, plasma oestradiol-binding capacity, at a dilution of 1:400, was 65.4±1.3% in the oestradiol-immunised group (H) and 64.9±2.3% in the combined immunisation group (I) compared to a preimmunisation non specific binding of < 4% inboth groups. Like the inhibin-binding capacity, the oestradiol-binding capacity then showed a steady decline ($p < 0.001$) which did not differ significantly between the two groups, falling to 61.6±4.2 and 58.2±1.7% by 48h and in the oestradiol-immunised (H)and combined immunisation (I) groups respectively.

8.3.2. Progesterone

The mean peripheral concentrations of progesterone in the 9 groups over the experimental period are shown in Fig. 8.1. Prior to treatment all groups showed normal luteal phase concentrations of progesterone, this concentration ranging from 2.7±0.7 to 3.4±0.4 ng/ml. In the Ovx group (A) there was a significant ($p < 0.001$) decline in the peripheral concentration of progesterone following ovariectomy, the concentration falling from 3.3±0.3 to 0.4±0.1ng/ml by 12h following ovariectomy. In the four groups treated with progesterone implants (B), (C), (D) and (E) there was a small though significant ($p < 0.01$) decline in the peripheral concentration of progesterone following treatment. However, in these four groups the mean concentration of progesterone over the experimental period did not differ from that in the control group, and was maintained above 2 ng/ml at all time points except at 48h in Group B where it fell to 1.9±0.2ng/ml. In the control group (F) and the three immunised groups (G, H and I) there was no significant change in the peripheral concentration of progesterone over the experimental period.

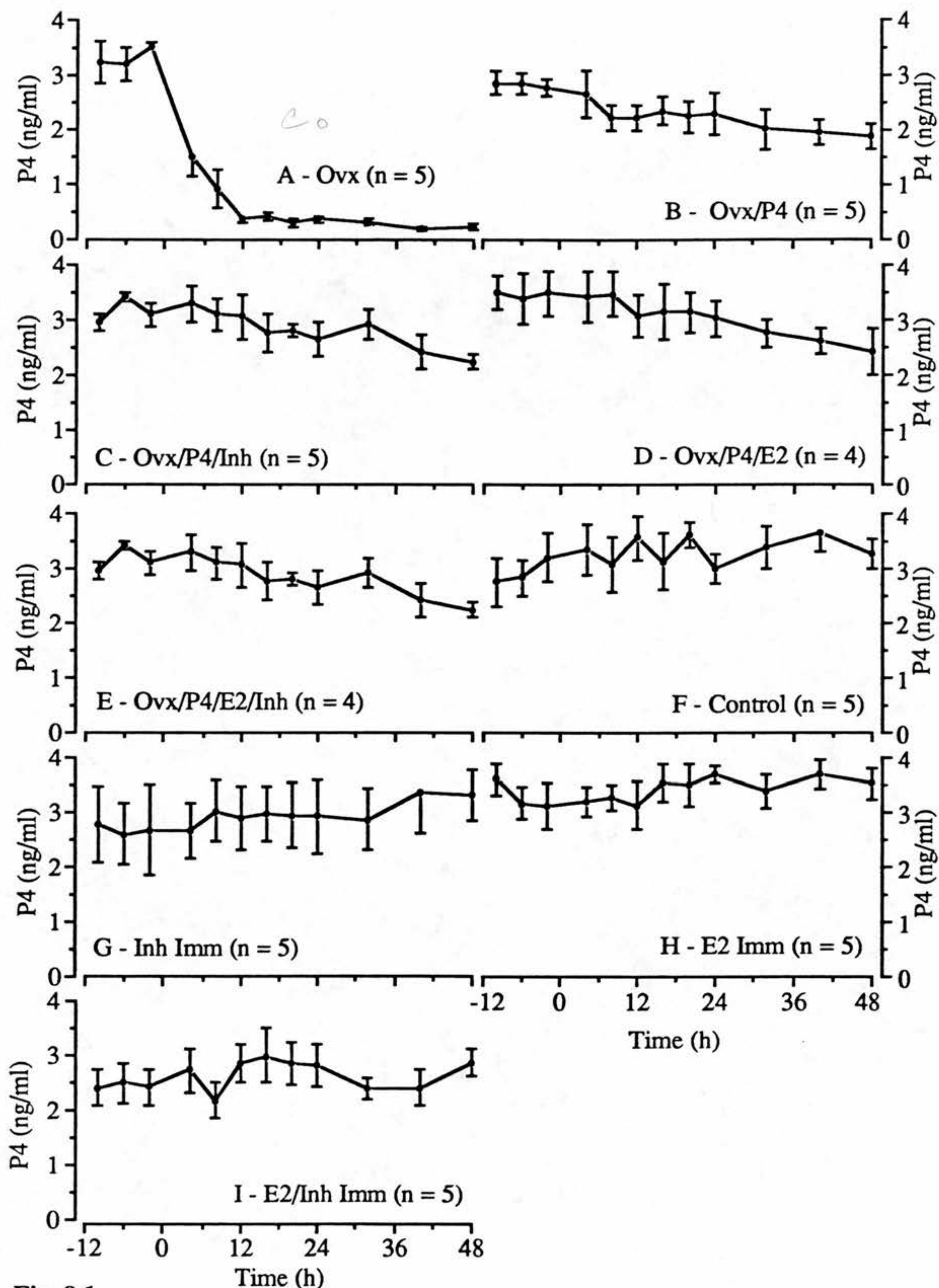

Fig. 8.1
Mean (± S.E.M) peripheral concentration of progesterone in groups of ewes that were either ovariectomised alone (a), ovariectomised and treated with progesterone (b), ovariectomised treated with progesterone and treated with inhibin (c) oestradiol (d) or oestradiol and inhibin (e), injected with control normal sheep plasma (f) or immunised against inhibin (g) oestradiol (h) or inhibin and oestradiol on day 10 of the luteal phase (time = 0h)

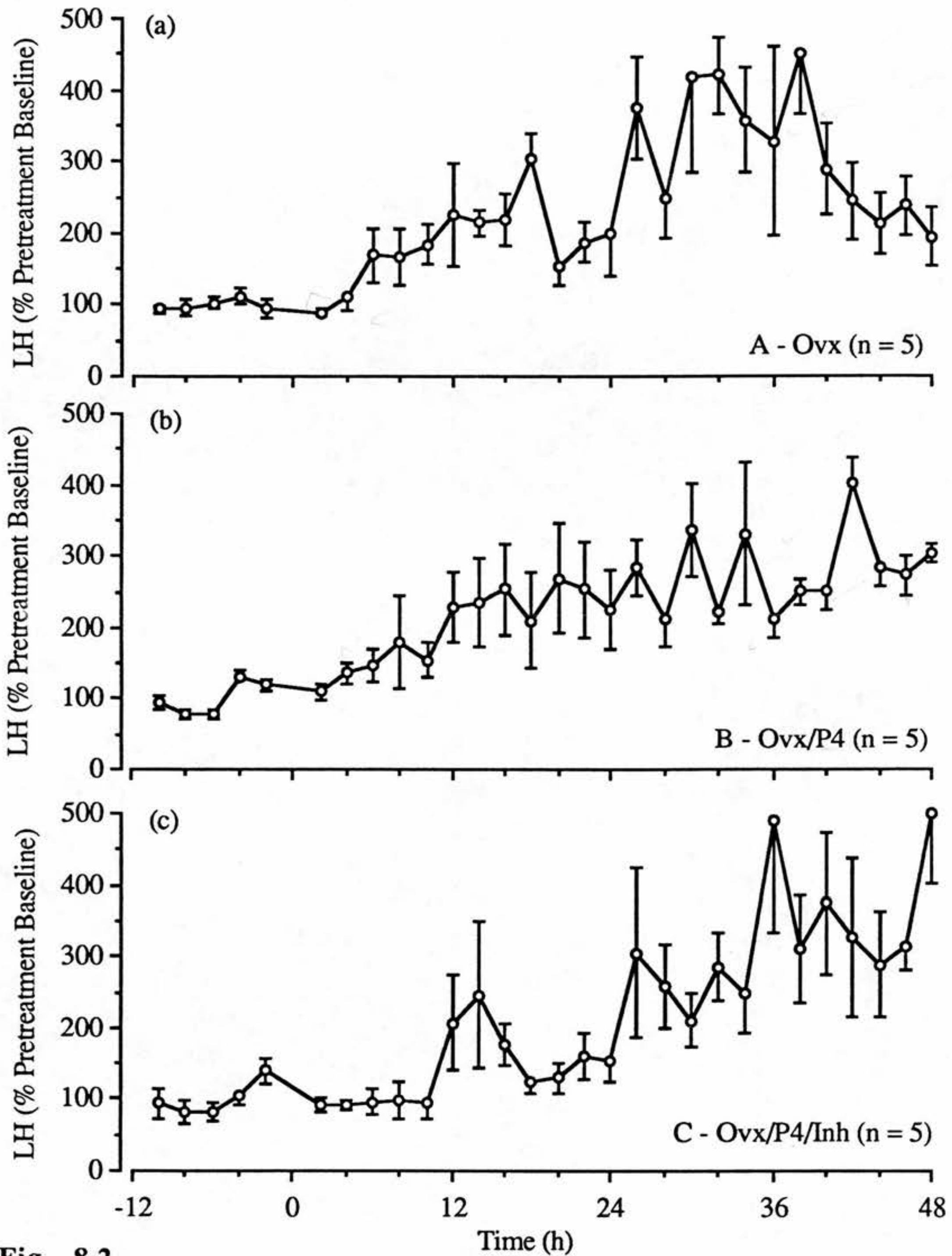

Fig. 8.2
Mean (±S.E.M.) peripheral concentrations of LH as a percentage of the mean concentration prior to treatment in groups of ewes either (a) ovariectomised alone, (b) ovariectomised and treated with progesterone or (c) ovariectomised and treated with progesterone and inhibin on day 10 of the luteal phase (time = 0h).

8.3.3. Luteinizing Hormone

Before treatment there were large between group variations in the mean peripheral LH concentration, values ranging from 1.7 ± 0.2 to 3.5 ± 0.4 ng/ml. As a result of this it was necessary to convert data to a percentage of the mean pretreatment concentration within each group to allow valid comparisons to be made between groups.

The concentrations of LH, as a percentage of pretreament levels, in the 9 groups are shown in Figs. 8.2, 8.3 and 8.4. Following ovariectomy there was a significant ($p < 0.001$) rise in the concentration of LH (Fig. 8.2a) which was not prevented by treatment with progesterone either alone (Fig. 8.2b) or in combination with inhibin (Fig. 8.2c). In the ovariectomised group treated with progesterone and oestradiol (Fig. 8.3a) this rise in LH concentration was prevented, and in the group treated with progesterone, oestradiol and inhibin (Fig. 8.3b) the rise was greatly attenuated, only a small though significant ($p < 0.05$) rise being seen. However, in both these groups the LH concentration over the experimental period did not differ significantly from that seen in the control group (Fig. 8.3c). In the group immunised against inhibin (Fig. 8.4a) LH concentration over the experimental period did not differ significantly from the control group. There was a significant elevation in the concentration of LH in the group immunised against oestradiol alone (Fig 8.4b; $p < 0.01$) and the group immunised against oestradiol in combination with inhibin (Fig. 8.4c; $p < 0.05$), and in both these groups the mean concentration of LH following treatment was significantly ($p < 0.01$) higher than in the control group.

LH pulse frequency and amplitude are shown in Fig. 8.5. LH pulse frequency in the control ovariectomised group (A) was significantly ($p < 0.001$) higher than in the Control Group (F) as well as significantly ($p < 0.05$) higher than in the ovariectomised groups treated with progesterone alone (B) or in combination with inhibin (C). The LH pulse frequency was in turn significantly higher than in the control group (F) in the ovariectomised groups treated with progesterone (B; $p < 0.01$) or progesterone and inhibin (C; $p < 0.05$), while in the two ovariectomised groups treated with oestradiol and progesterone, either with (E) or without (D) inhibin LH pulse frequency did not differ significantly from the control group. In the groups immunised against inhibin (G) oestradiol (H) or inhibin and oestradiol (I) the pulse frequency did not differ significantly from the control group (F).

LH pulse amplitude was highly variable within groups resulting in a lack of statistical significance between groups. LH pulse amplitude was higher in the control ovariectomised (A) and progesterone treated - ovariectomised (B) groups than in the control group, though these difference were not statistically significant. In the

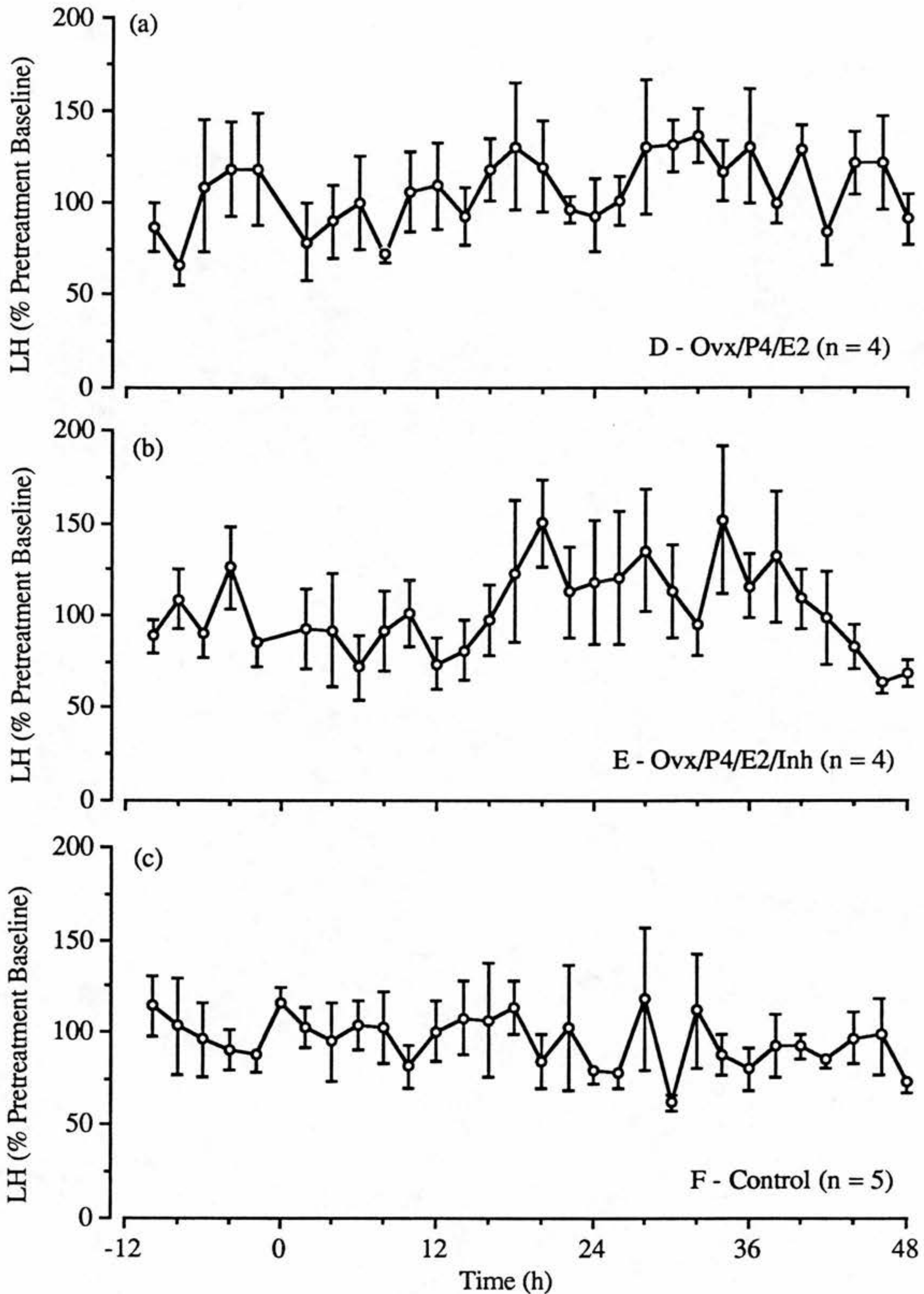

Fig. 8.3
Mean (±S.E.M.) peripheral concentrations of LH as a percentage of the mean concentration prior to treatment in groups of ewes that were either ovariectomised, treated with progesterone and treated with oestradiol (a) or oestradiol and inhibin (b), or injected with control sheep plasma (c) on day 10 of the luteal phase (time = 0h).

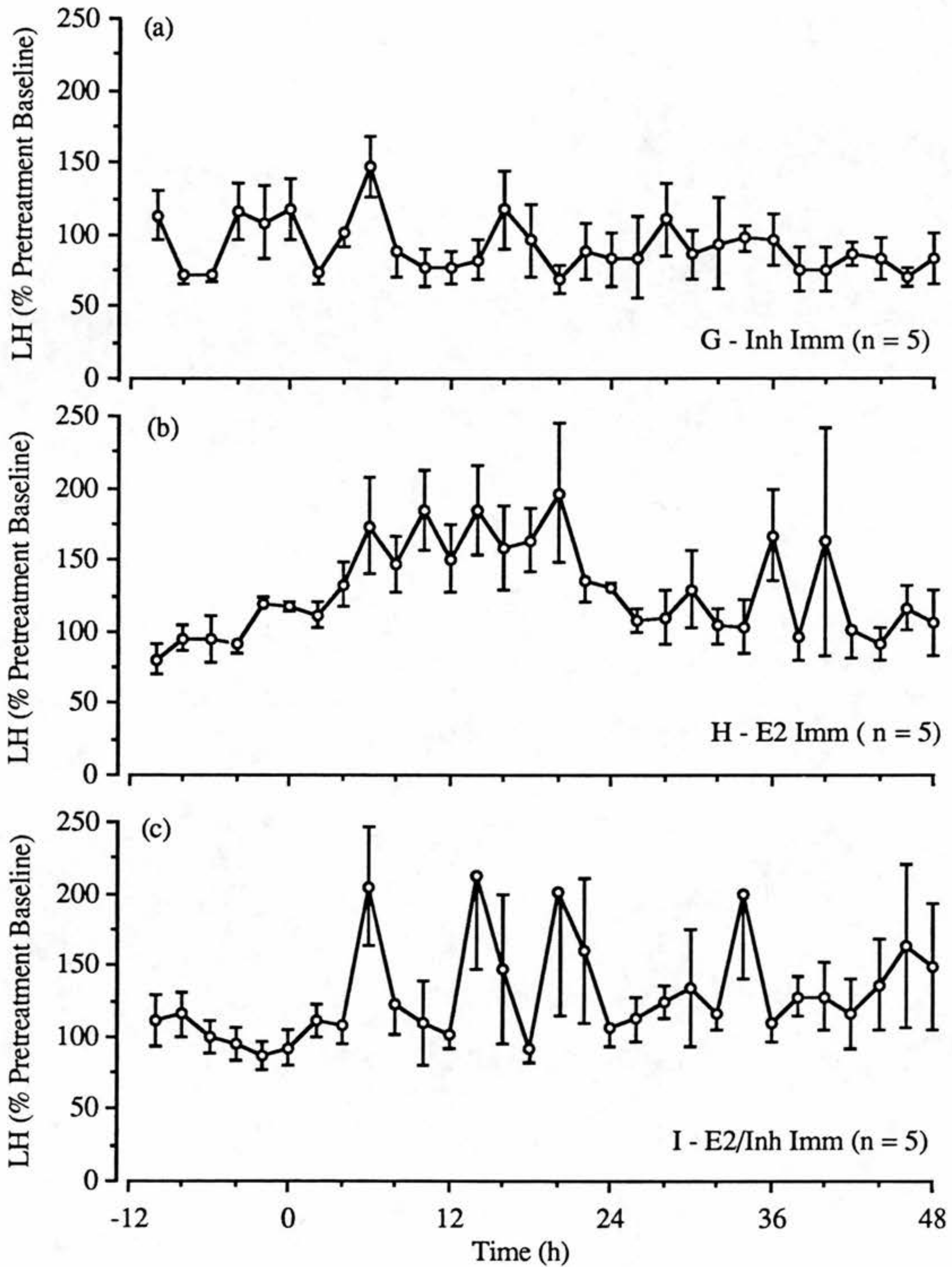

Fig. 8.4
Mean (±S.E.M.) peripheral concentrations of LH as a percentage of the mean concentration prior to treatment in groups of ewes immunised against (a) inhibin, (b) oestradiol or (c) oestradiol and inhibin on day 10 of the luteal phase (time = 0h).

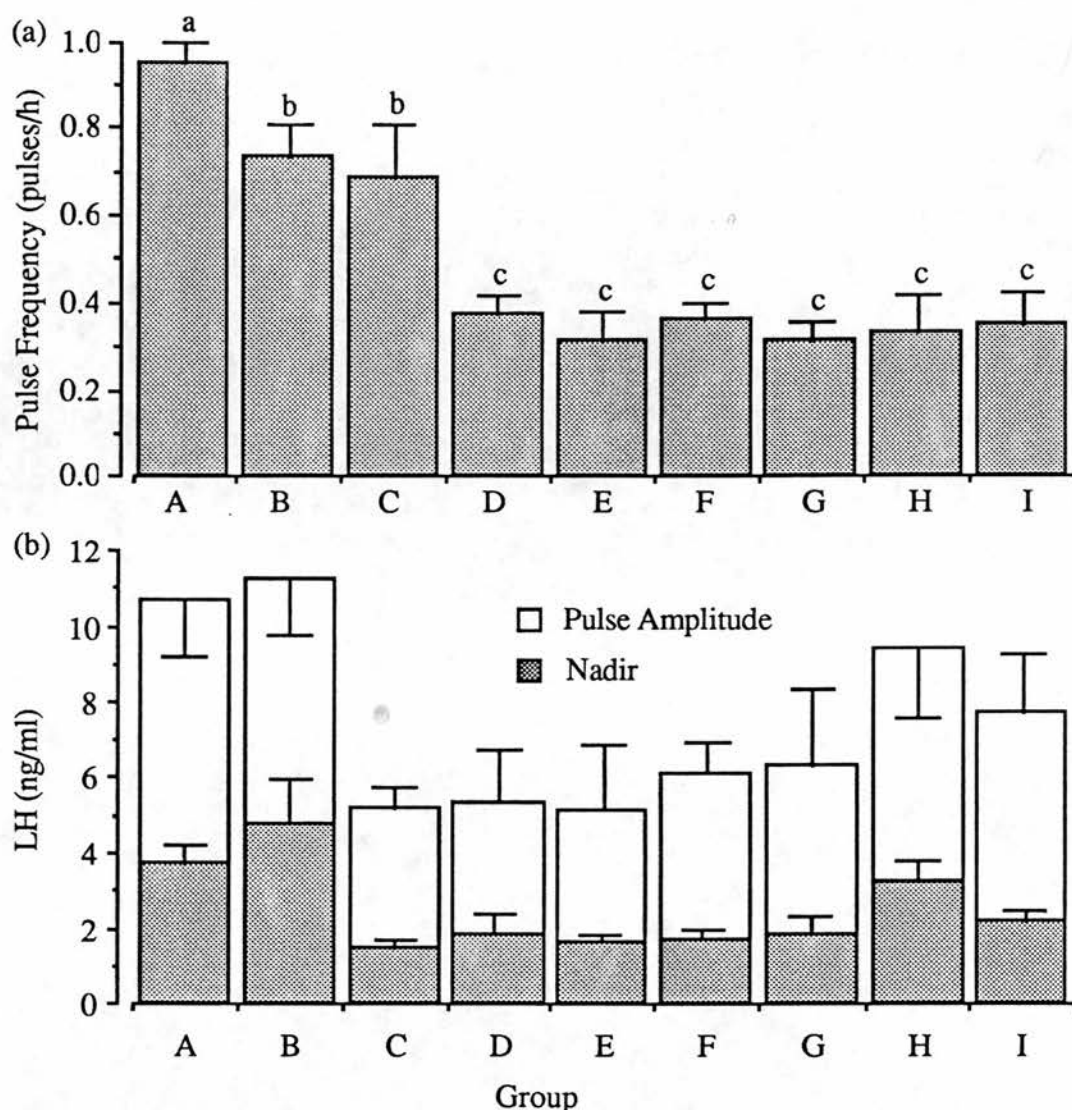

Fig. 8.5
(a) mean (±S.E.M.) LH pulse frequency and (b) mean (±S.E.M.) LH pulse amplitude (□) and nadir (▩) during the period from 12 to 24h following treatment in groups of animals either ovariectomised alone (A), ovariectomised and treated with progesterone (B), ovariectomised treated with progesterone and treated inhibin (C), oestradiol (D) or oestradiol and inhibin (E), injected with control sheep plasma (F), or immunised against inhibin (G), oestradiol (H) or oestradiol and inhibin (I) on day 10 of the luteal phase (time = 0h). (Groups D and E n = 4; other groups n = 5).
(Note: in Group C mean LH concentration prior to treatment was significantly ($p < 0.05$) lower than in the other groups and so a relatively low pulse amplitude would be expected) (Bars with different letters are significantly different ab, bc $p < 0.05$ ac $p < 0.01$ student's unpaired t-test)

ovariectomised group treated with progesterone and inhibin (C) pulse amplitude was significantly ($p < 0.05$) lower than in groups A or B. However, in this group treated with inhibin the mean pretreatment LH concentration was also significantly ($p < 0.01$) lower than in the other two groups and so a lower pulse amplitude would be expected. In the ovariectomised groups treated with progesterone and oestradiol with (E) or without (D) inhibin LH pulse amplitude was similar to the control group (F). LH pulse amplitude was elevated in the two groups immunised against oestradiol either alone (H) or with inhibin (I) compared to the control group, though not significantly so, while in the group immunised against inhibin alone (G) pulse amplitude was similar to the control group (F).

8.3.4. Follicle Stimulating Hormone

Mean peripheral concentrations of FSH in the 9 groups during the experimental are shown in Figs. 8.6 and 8.7. Due to differences in the mean pretreatment concentration of FSH between groups, concentrations of FSH were converted to a percentage of these pretreatment values to allow an accurate comparison to be made of differences in response between groups, and these percentage responses are shown in Figs. 8.8 and 8.9.

Following ovariectomy there was a significant rise in the peripheral concentration of FSH in the control ovariectomised group (A; $p < 0.001$), and in the ovariectomised groups treated with progesterone either alone (B; $p < 0.001$), or in combination with inhibin (C; $p < 0.001$) or oestradiol (D; $p < 0.05$), while in the group treated with progesterone, oestradiol and inhibin (E) there was no significant change in the peripheral concentration of FSH (Fig. 8.6). In the control group (F) there was no significant change in the peripheral concentration of FSH, while FSH showed a significant rise following treatment in the inhibin immunised group (G; $p < 0.001$), the oestradiol immunised group (H; $p < 0.01$) and the combined immunised group (I; $p < 0.001$) (Fig 8.7)

The magnitude of the rise in FSH concentration in the progesterone - treated ovariectomised group (B) did not differ significantly from that seen in the control ovariectomised group (A), the responses in both these groups differing significantly ($p < 0.001$) from the control group (Fig. 8.8). In the ovariectomised groups treated with inhibin (C) or oestradiol (D) the rises in FSH, though significantly ($p < 0.001$) greater than the control group, were significantly lower than those seen in group A ($p < 0.001$) or Group B ($p < 0.01$). In the ovariectomised group treated with inhibin and oestradiol (E) the concentration of FSH did not differ significantly from the control group (F). In

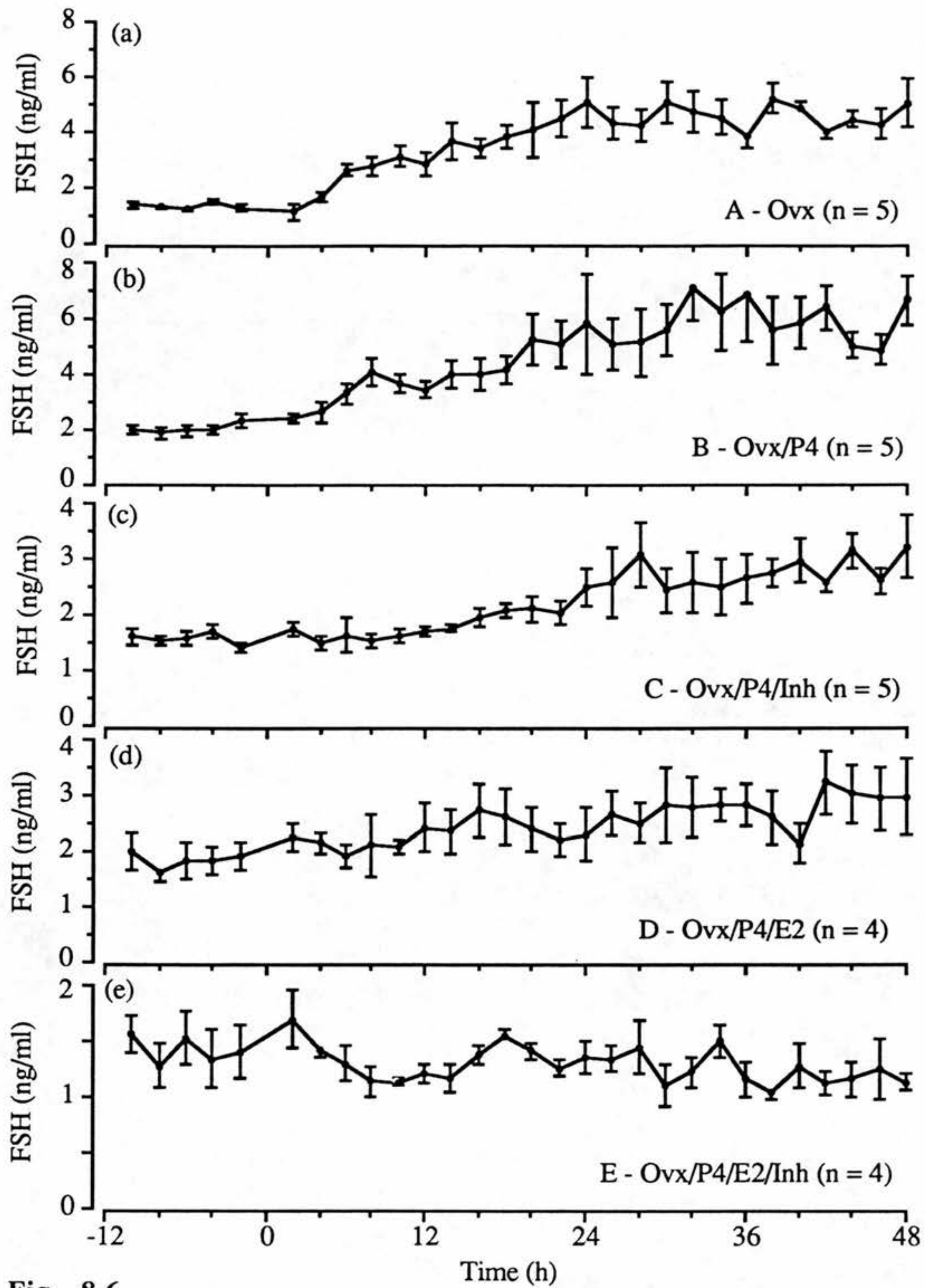

Fig. 8.6
Mean (± S.E.M) peripheral concentration of FSH in groups of ewes that were either ovariectomised alone (a), ovariectomised and treated with progesterone (b) or ovariectomised treated with progesterone and treated with inhibin (c) oestradiol (d) or oestradiol and inhibin (e) on day 10 of the luteal phase (time = 0h)

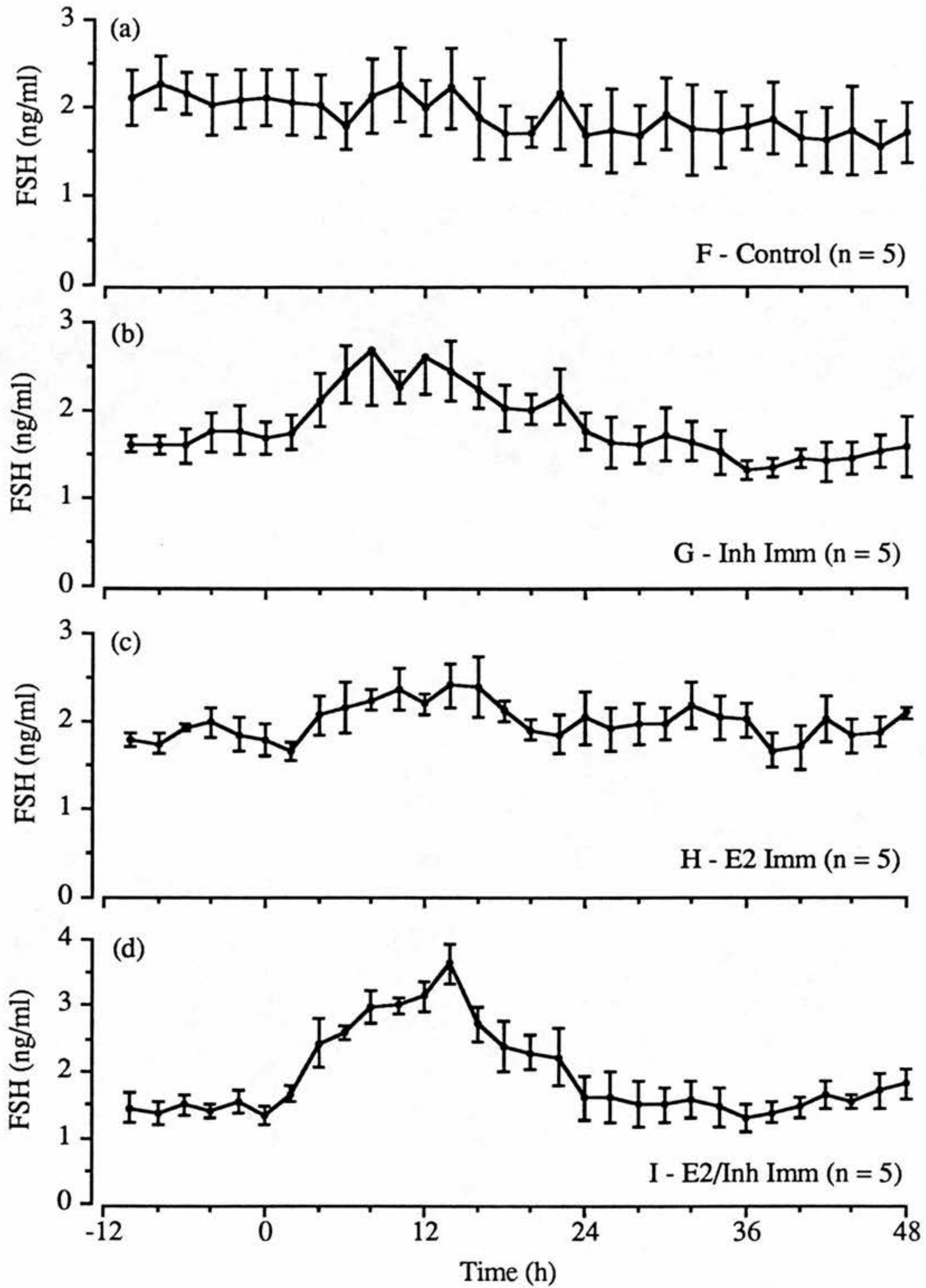

Fig. 8.7
Mean (± S.E.M) peripheral concentration of FSH in groups of ewes treated with (a) control sheep plasma, (b) inhibin antibody, (c) oestradiol antibody or (d) inhibin and oestradiol antibodies on day 10 of the luteal phase (time = 0h)

the groups immunised against inhibin (G) or oestradiol (H) individually the rise in FSH concentration differed significantly ($p < 0.05$) from the control group (Fig. 8.9), while the rise in FSH concentration in the combined immunisation group (I) was significantly ($p < 0.01$) larger than that seen in either of the individual immunisation groups. The time to the onset of the rise in FSH, the duration of this rise and the mean elevation of FSH concentration over this period for the 3 immunised groups are shown in Table 8.1. The delay from immunisation to the onset of the response showed no significant differences between groups, while the duration of the response was significantly ($p < 0.05$) lower in the oestradiol immunised than in the combined immunisation group. The magnitude of the rise was significantly larger in the combined immunisation group than in groups immunised against inhibin ($p < 0.01$) or oestradiol ($p < 0.001$) alone.

Table 8.1 Characteristics of the FSH responses in the three immunised groups

Group	Time to Response	% Rise	Duration
Inhibin (G)	4.0 ± 0.6 h	35.6 ± 6.0	18.4 ± 0.5 h
Oestradiol (H)	4.8 ± 1.0 h	20.8 ± 7.5	15.4 ± 0.8 h
Combined (I)	3.6 ± 0.7 h	78.8 ± 7.2	22.8 ± 2.3 h

Over the 12h period immediately following treatment the rise in FSH concentration in the group immunised against inhibin and oestradiol did not differ significantly from that seen in the control ovariectomised group (Fig. 8.9) or the ovariectomised group treated with progesterone, but was significantly ($p < 0.01$) greater than that seen in the ovariectomised groups treated with inhibin or oestradiol. Finally, the responses in FSH seen following the individual immunisations did not differ significantly from the responses seen in the ovariectomised groups treated with progesterone and inhibin (C) or progesterone and oestradiol (D).

8.5. Discussion

In this study we have shown that passive immunisation against either inhibin or oestradiol during the luteal phase results in an increase in the peripheral concentration of FSH, and that passively immunising ewes against the two hormones in combination results in a much larger rise in FSH concentration. These results demonstrate that the

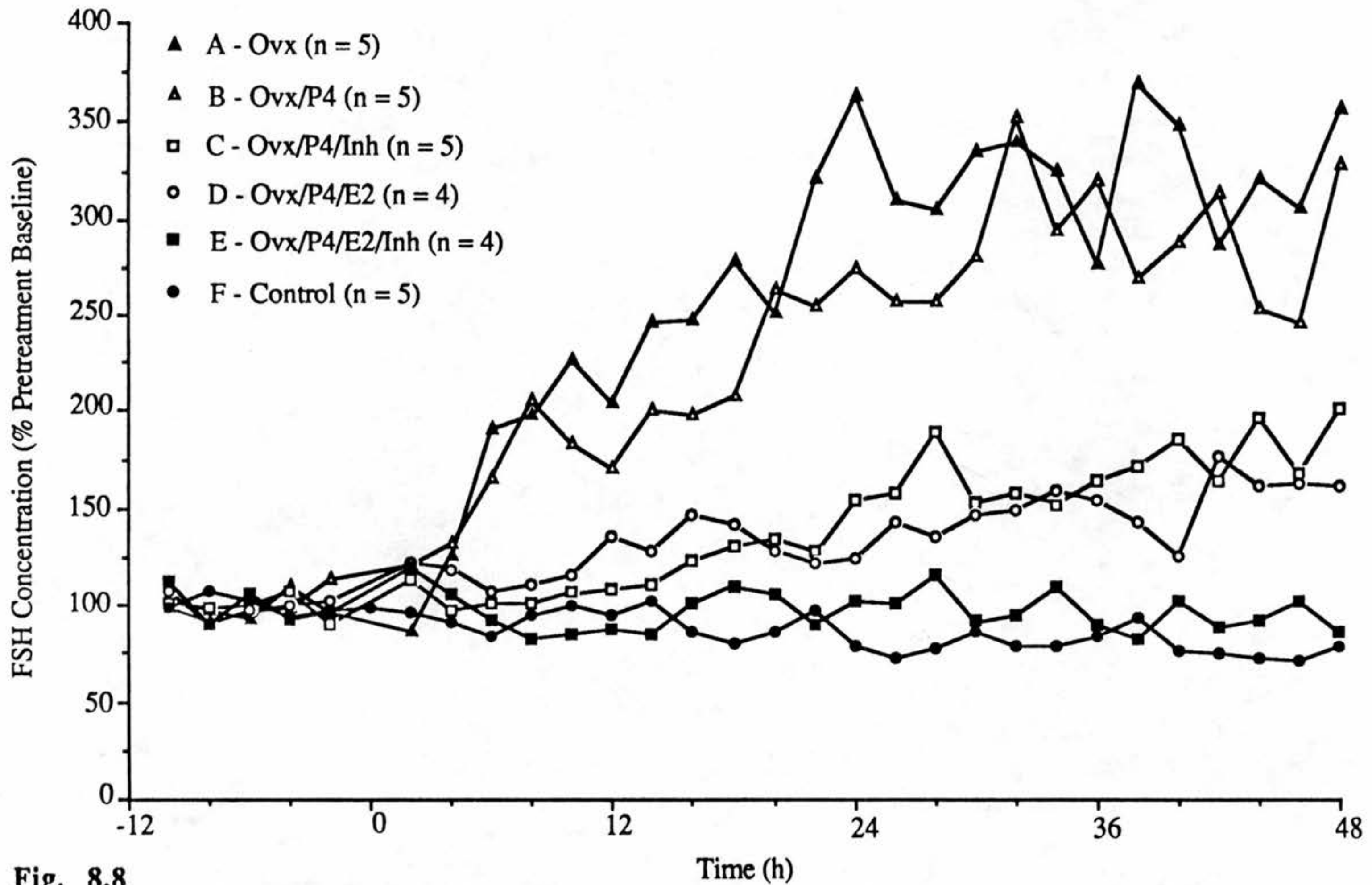

Fig. 8.8
Mean concentration of FSH as a percentage of the mean value prior to treatment in groups of animals ovariectomised alone (A), ovariectomised and treated progesterone and different combinations of oestradiol and inhibin (B, C, D & E), or injected with control sheep plasma (F) on day 10 of the luteal phase (time = 0h)

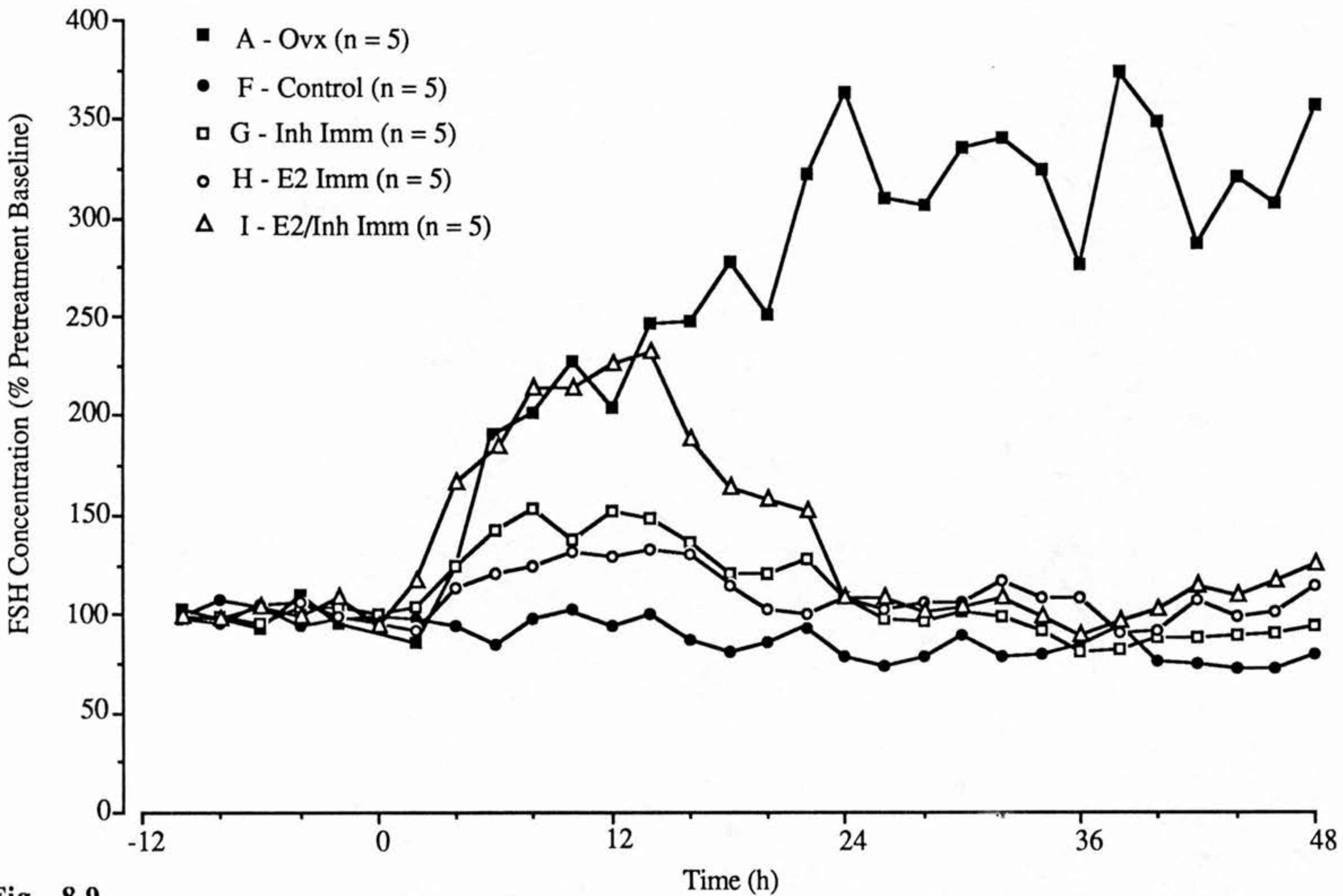

Fig. 8.9
Mean concentration of FSH as a percentage of the mean value prior to treatment in groups of animals either ovriectomised (A), treated with control sheep plasma (F), or immunised against inhibin (G), oestradiol (H) or inhibin and oestradiol (I) on day 10 of the luteal phase (time = 0h)

two hormones are acting together in the physiological control of FSH secretion at this time. We have also demonstrated that oestradiol and inhibin, when administered to acutely ovariectomised ewes in "physiological" quantities, are able to maintain peripheral FSH concentrations within a normal range. This result confirms the finding of a similar study performed by Martin et al. (1988) in which the post - castration rise in FSH concentration was prevented by the use of a regime of oestradiol implants and follicular fluid injections similar to that used in this study. LH is under the dual control of oestradiol and progesterone, and in this study the doses of oestradiol and progesterone used were able to maintain basal LH concentrations, as well as LH pulse frequency and amplitude within the normal physiological range following ovariectomy. This suggests that the oestradiol implants used were supplying oestradiol at a rate similar normal ovarian output. In the experiment described in Chapter 5 it was found that a dose of 1ml/8h of the same pool of steroid - stripped follicular fluid as that used in this experiment was sufficient to generate peripheral plasma concentrations of inhibin at the top of the physiological range. This would suggest that the dose of inhibin used in this study (i.e. 0.8ml oFF/8h) was probably appropriate. While we were unable to measure inhibin concentrations in the plasma of the animals in this study, the fact that the dose of inhibin used combined with an apparently phisiological dose of oestradiol to maintain normal FSH concentrations after ovariectomy would suggest that the dose of inhibin used was approximately equivalent to normal ovarian output.

The fact that the provision of physiological quantities of oestradiol and inhibin was sufficient to maintain peripheral FSH concentrations following ovariectomy suggests that these two hormones are the major factors involved in the control of FSH secretion. However, progesterone was also administered to these animals and so a role for this hormone cannot be excluded. In the group immunised against inhibin and oestradiol in combination, the initial rise in FSH concentration matched very closely that seen following ovariectomy. As FSH levels showed a rise similar to the post - castration rise in this immunised group in the presence of luteal phase progesterone concentrations this would suggest that the action of inhibin and oestradiol can account fully for the physiological suppression of FSH secretion, and that no other factors, including progesterone, play a significant role in this process.

Over the 48h period following treatment the groups immunised against inhibin or oestradiol alone showed similar responses in the concentration of FSH, while in the two ovariectomised groups treated with either inhibin or oestradiol the degree to which the post - castration rise in FSH secretion was suppressed was similar. These facts, together with the fact that over the 12h period following treatment these four groups all showed statistically similar rises in the concentration of FSH would suggest that during

the luteal phase inhibin and oestradiol are of similar importance in the control of FSH secretion.

The finding in this study that individual inhibin immunisations both resulted in a rise in FSH concentration while a combined immunisation against both hormones resulted in a significantly larger rise is similar to the result obtained when the same approach was used in the follicular phase (Chapter 7). One difference between the two stages of the cycle was that during the follicular phase the rise in FSH concentration following immunisation against oestradiol resulted in a larger and more prolonged elevation in FSH concentration (43% rise over 44h during the follicular phase compared with 21% over 15h during the luteal phase). This is probably a reflection of the consistently high levels of oestradiol found during the follicular phase compared with much lower and more variable levels during the luteal phase, and suggests oestradiol may be playing a more important role in controlling FSH secretion during the follicular phase. This idea is supported by the fact that immunisation against inhibin, the peripheral concentration of which shows less variation through the oestrous cycle than oestradiol, resulted in a rise in FSH concentration in this experiment of similar magnitude and duration to that seen following immunisation during the follicular phase. In the experiment described in Chapter 6 immunisation against inhibin during the luteal phase resulted in a rise in FSH concentration of greater magnitude and duration than that seen in this experiment. This difference is difficult to explain, but may have been the result of immunisation being carried out on day 8 of the cycle in the earlier experiment compared to day 10 in this experiment. A further difference in the two experiments was that the immunisation described in Chapter 6 was carried out earlier in the breeding season than in this experiment (November compared with January). It should, however, be noted that the differences in the magnitude of the response of FSH to inhibin immunisation in the two luteal phase experiments was relatively small when compared to the large differences in the responses to oestradiol immunisation between the two stages of the cycle, and also that there was considerable overlap in both the magnitude and duration of the response in individual animals between the two luteal phase immunisation experiments.

In this experiment, treatment with progesterone implants which maintained normal luteal phase progesterone concentrations had no effect on the post - castration rise in the peripheral concentration of LH. This finding is in keeping with that of Martin et al. (1988) while Karsch et al. (1977) found that a similar dose of progesterone was sufficient to prevent this rise in LH concentration. The experiment performed by Martin et al. involved ovariectomy between days 13 and 15 of the cycle while in the experiment of Karsch et al. ovariectomy was performed on day 2 after oestrus.

Oestradiol priming has been shown to increase the ability of progesterone to suppress LH secretion (Goodman et al., 1980), and when ovariectomy was performed early in the luteal phase the hypothalamus was probably still "sensitised" to the action of progesterone, while later in the luteal phase this effect would have diminished resulting in progesterone being ineffective in preventing the rise in LH secretion following ovariectomy. In the experiment of Karsch et al. (1977) progesterone was only able to maintain LH concentrations for 48h, supporting this suggestion. In this experiment treatment with progesterone alone resulted in a significant decrease in LH pulse frequency in ovariectomised ewes. Goodman & Karsch (1980) found a much larger decline in LH pulse frequency, but as suggested previously, this probably was once again due to the differences in the timing of the ovariectomies.

A feature of this experiment which requires explanation is the fact that in ovariectomised ewes in which normal progesterone levels had been restored (Group B) LH pulse frequency was significantly higher than that seen in the control group, while in intact ewes in which oestradiol had been immunoneutralised no such elevation was evident. Immunoneutralisation involves an equilibrium between bound and free hormone, with a certain amount of free hormone invariably present. LH pulse frequency was measured between 12 and 24h following immunisation, and it is possible that by this time an increase in ovarian oestradiol production may have ocurred in response to the oestradiol immunisation, increasing the amount of free oestradiol present. This free oestradiol would then be able to enhance the ability of progesterone to maintain LH pulse frequency, while in the ovariectomised animals treated with progesterone virtually all oestradiol would have cleared from the circulation by this time.

In conclusion, the results of this study indicate that the actions of oestradiol and inhibin are sufficient to account fully for the control of FSH secretion in the ewe, and show that during the luteal phase these two hormones may be of similar importance in this process. The results also confirm that oestradiol and progesterone are the main hormones involved in the control of LH secretion, and that inhibin does play any significant role in the physiological control of LH secretion.

Chapter 9

Passive Immunisation against Inhibin and Oestradiol in Ewes with an Ovary Transplanted to the Neck

9.1. Introduction

The experiments on passive immunisation performed in Chapters 6, 7 and 8 have demonstrated that immunisation against inhibin and/or oestradiol results in a marked elevation in the peripheral concentration of FSH during both the luteal and the follicular phases of the oestrous cycle. However, in all cases this rise in FSH was transitory, concentrations of FSH returning to those seen prior to immunisation within 17 to 29h of immunisation with antibodies against inhibin and within 40h of immunisation with antibodies against oestradiol. In the ewes used in this experiment one ovary and its vascular pedicle had been transplanted to a site in the neck (Goding, et al., 1967) allowing repeated sampling of ovarian venous blood in the conscious animal. By measuring hormone secretion rates directly from the ovary after immunisation it was hoped that useful information could be obtained on the compensatory mechanism causing this fall in FSH seen following the post - immunisation rise.

Rises in the peripheral concentration of FSH following PMSG treatment (Cahill & Dufour, 1979; McNatty et al, 1982), infusion of FSH (McNeilly, 1985; Henderson et al., 1988) or during the "rebound" rise in FSH concentration following cessation of bFF treatment (Henderson et al., 1986; Wallace & McNeilly, 1985) all promote follicle growth, and result in an increase in ovulation rate if treatment is undertaken at an appropriate time in the oestrous cycle. Cross - sectional studies have been used to investigate the changes in follicle populations following such treatments (Dott et al., 1979), but longitudinal studies have not been possible. In horses (Palmer, 1987) and cattle (Pierson & Ginther, 1987a,b; Sirois & Fortune, 1988) longitudinal studies of follicle development during the oestrous cycle have been performed using ultrasound scanners fitted with intra-rectal probes. In the ewes used in this study an ovary was situated under the skin in the neck in a site ideally suited to ultrasound scanning, and by using this technique it was possible to monitor the changes in follicle populations at different times in the same animal during the rise in FSH concentration resulting from immunisation against inhibin and oestradiol.

9.2. Materials and Methods

9.2.1. Experimental Procedure

The following experimental procedures were carried out during the breeding season, in February and March, on 14 mature Finn x Merino ewes in which the left ovary and its vascular pedicle had been transplanted to the neck in order to allow ovarian venous blood sampling in the conscious animal (see Section 2.1). Oestrous cycles were synchronized (see Section 2.2) and on day 9 of the luteal phase animals cannulated (see Section 2.4) and then allocated three groups:

1. Control - 10ml normal sheep plasma (n = 5)
2. Inhibin Immunised - 10ml inhibin antibody (n = 5)
3. Oestradiol Immunised - 10 ml oestradiol antibody (n = 4)

All plasma/antibody treatments were given on day 10 of the luteal phase of the oestrous cycle by means of a single i.v. bolus injection. Samples of jugular and timed ovarian venous blood were collected at 4h intervals from 12h before until 48h after treatment, and ovarian secretion rates calculated after correction for the haematocrit (Collet et al., 1973). From 12 to 20h following treatment jugular and ovarian venous blood samples were collected at 15 min intervals to permit monitoring of pulsatile hormone release. Jugular samples were assayed for; FSH, LH, progesterone and inhibin while ovarian samples were assayed for oestradiol, androstenedione and inhibin. Plasma inhibin and oestradiol binding capacities were also determined in jugular plasma.

9.2.2. Ultrasound Scanning

The follicle populations of the animals were monitored 24h before treatment, and at 12h intervals from treatment until the end of the experiment using a combined sector real time ultrasound scanner (DSL 300) with a sector 7.5 MHz transducer probe (Diagnostic Sonar, Kirkton Campus, Livingstone, Scotland). Prior to scanning the area of skin over the ovary was clipped and scanning gel (Siel Sound Gel: Siel Imaging Equipment Ltd, Aldermasten, Berkshire, U.K.). The ovary was scanned in both the vertical and horizontal planes, and the diameter of the antral cavity and the position of all follicles > 2mm measured in the medial/lateral, dorsal/ventral and cranial/caudal planes.

9.2.3. Analysis of Data

Follicle numbers at scanning were analysed on data that had been transformed ($\sqrt{x}+^{1}/_{2}$) due to the presence of no follicles of a particular class at certain time points in some animals. Peripheral hormone concentrations and ovarian hormone secretion rates were analysed by repeated sample analysis of variance on data that had been ln transformed due to the large variation in hormone concentrations between animals. Parameters of pulsatile hormone secretion were analysed using the Munro pulse analysis programme and specific parameters compared using student's unpaired t - tests. Mean rates of ovarian hormone secretion were determined by calculating the average secretion rate over the period of intensive blood sampling from 12 to 20h after antibody administration, and analysis was then performed on ln transformed data using a student's unpaired t - test.

9.3. Results

9.3.1. Plasma Hormone Binding Capacity

Following immunisation there was an increase ($p < 0.001$) in the ability of plasma to bind inhibin and oestradiol in all the immunised animals. In the animals immunised against inhibin, by 4h following immunisation, plasma inhibin-binding capacity, at a dilution of 1:15, was 71.4±2.3 % (± S.E.M.) compared to a pre-immunisation non specific binding of 7.2±0.5%. The binding capacity then showed a steady decline ($p < 0.001$) over the course of the experiment in both groups, falling to 63.4±2.7% by 48h. In the oestradiol-immunised animals, by 2h following antibody injection, plasma oestradiol-binding capacity, at a dilution of 1:400, was 74.8±2.5% compared to a pre - immunisation non specific binding of 2.0±0.2%. Like the inhibin-binding capacity, the oestradiol-binding capacity then showed a steady decline ($p < 0.001$), falling to 59.3±2.8% by 48h.

9.3.2. Follicle Numbers

During the course of the experiment there was a small though significant ($p < 0.05$) increase in the total number of follicles detected per ovary (Fig. 9.1.). In contrast there was a large highly significant ($p < 0.001$) rise in the total number of follicles per ovary in both treatment groups following immunisation. In the animals treated with inhibin antibodies the total number of follicles per ovary rose from a mean of 5.5±1.0

before immunisation to a maximum of 13.6±1.4, while in the group immunised against oestradiol this rise was from 4.6±0.5 to 11.5±1.0. While the response seen in the group immunised against inhibin was larger than that in the oestradiol immunised group this difference was not significantly different. Fig 9.2 shows the proportions of small (< 3.5 mm) medium (3.5 - 4.5 mm) and large (> 4.5 mm) follicles per ovary at the various time periods. In the control group there was a small but significant ($p < 0.05$) rise in the number of small follicles while in the two immunised groups large highly significant ($p < 0.001$) rises in the number of small follicles were observed. In the group immunised against oestradiol the rise in the total number of follicles per ovary was due entirely to this rise in the number of small follicles while in the group immunised against inhibin there was also a rise in the number of medium sized follicles from 1.3±0.5 before to 3.2±1.0 by 48h after immunisation which approached statistical significance ($p < 0.07$). In the control and inhibin immunised groups there were some large follicles present at all time periods, while in the group immunised against inhibin, none of the four ewes possessed a large follicle at 24h following immunisation.

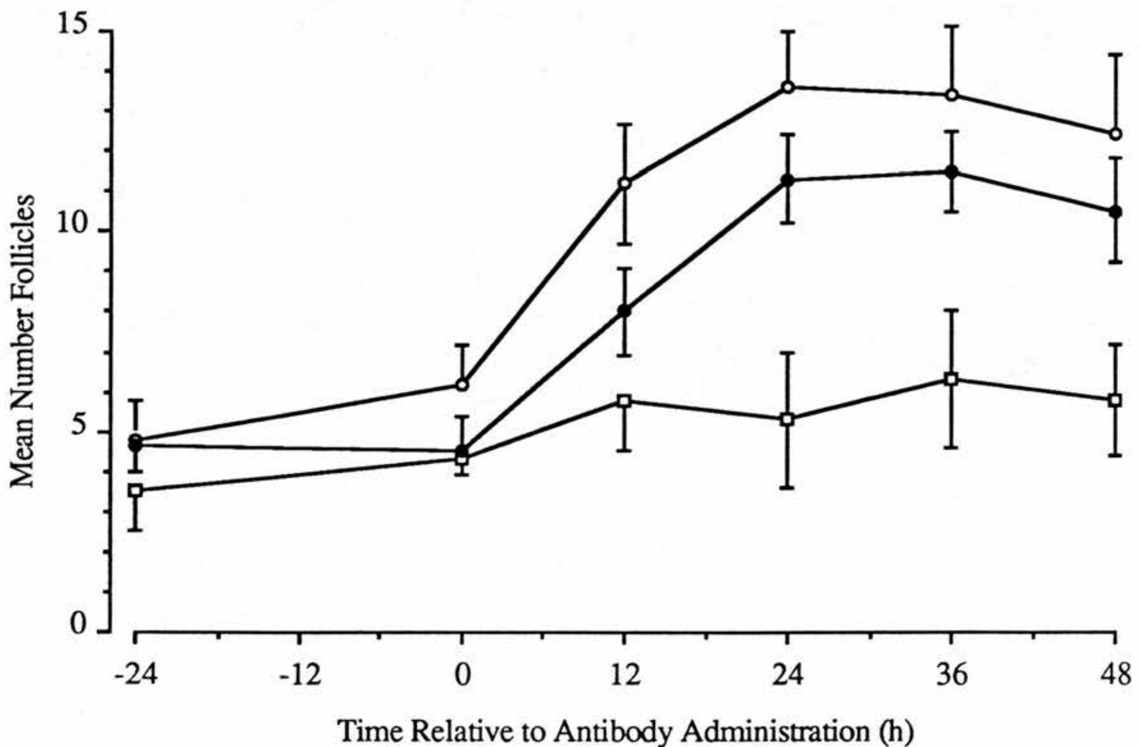

Fig. 9.1.
Mean (± S.E.M.) number of follicles per ovary in ewes injected at time 0h with a single i.v. bolus of either normal sheep plasma (□ ; n = 4), inhibin antibody (○; n = 5) or oestradiol antibody (●; n = 4).

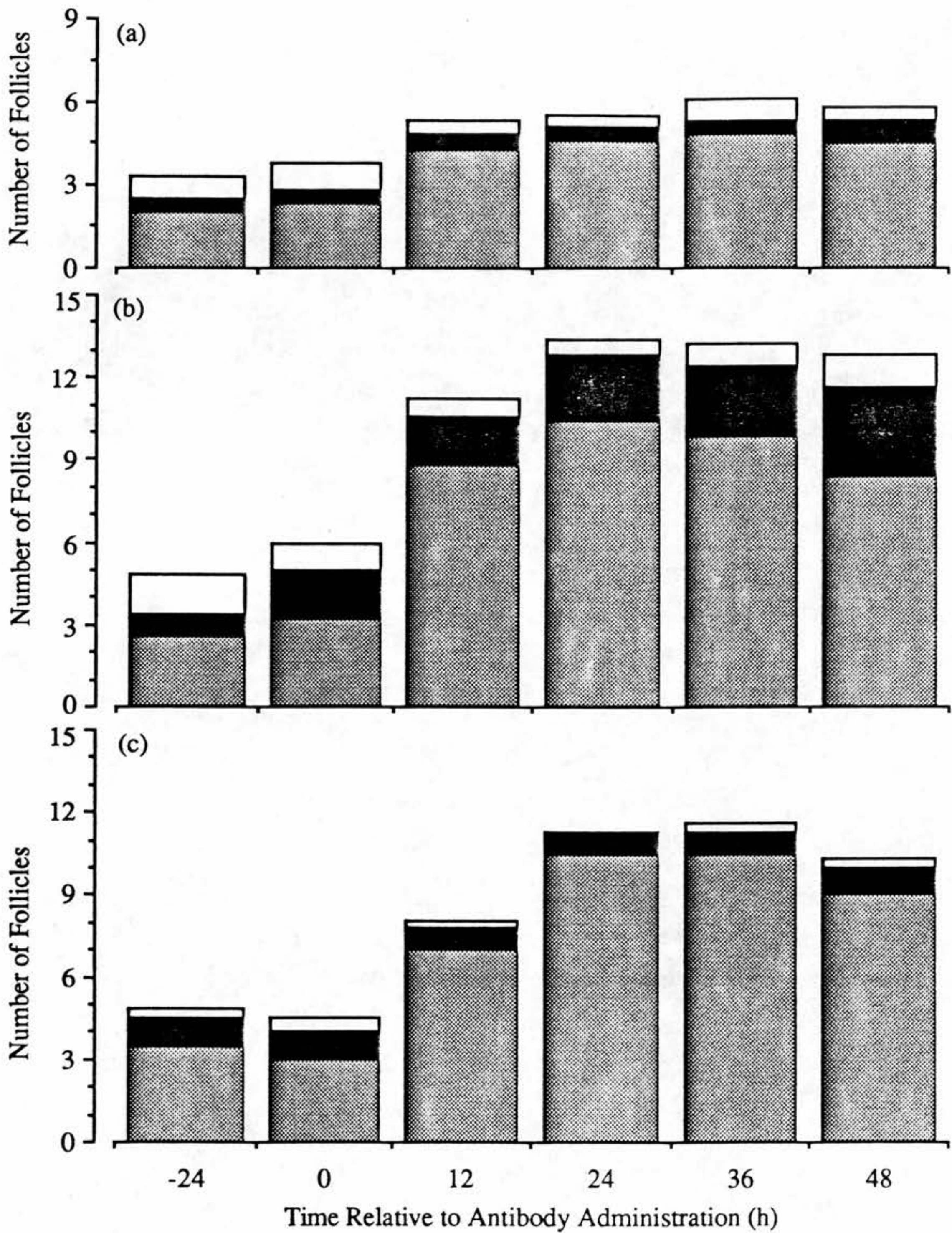

Fig. 9.2.
Mean number of follicles per ovary with a diameter of either < 3.5 mm (▩), 3.5 - 4.5 mm (■) or > 4.5 mm (□) in ewes injected at time 0h with a single i.v. bolus of either (a) normal sheep plasma (n = 4), (b) inhibin antibody (n = 5) or (c) oestradiol antibody (n = 4).

9.3.3. Peripheral Hormone Concentrations

Mean peripheral progesterone concentrations were 2.50±0.85 ng/ml in the control group, 2.79±0.25 ng/ml in the inhibin immunised group and 3.39±0.63 ng/ml in the oestradiol immunised groups, these concentrations being unaffected by treatments. In the control group the peripheral concentration of FSH remained unchanged while in the groups immunised against inhibin and oestradiol the mean FSH concentration rose significantly ($p < 0.001$) following treatment (Fig. 9.3.) The rise in FSH concentration in the group immunised against inhibin was significantly ($p < 0.05$) greater than that in the oestradiol immunised group, and resulted in a mean elevation in FSH concentration over the 24h period following immunisation, as a percentage of the pre-immunisation concentration, of 54.1±7.4% compared to 35.2±15.6% in the oestradiol immunised group.

In the group immunised against oestradiol there was a significant ($p < 0.01$) rise in the peripheral concentration of inhibin over the 12h period following immunisation, this rise in inhibin correlating positively with the rise in FSH seen at this time ($R = 0.94$; $p < 0.06$). Following treatment control inhibin concentrations showed no significant changes, though there was a significant ($p < 0.05$) decline prior to treatment.

The characteristics of pulsatile LH secretion during the period of intensive blood sampling from 12 to 20h are shown in Table 9.1. Due to the occurrence of only 2 LH pulse in the animals in the control group it was not possible to produce an estimate of

Table 9.1.

LH pulse characteristics in control ewes (n = 4) and ewes immunised against inhibin (n = 5) or oestradiol (n = 4) on day 10 of the luteal phase of the oestrous cycle. In the control group there were insufficient pulses to calculate mean amplitude or nadir.

	Control	Inhibin Immunised	Oestradiol Immunised
Pulse Frequency /8h (pulses/ewes)	0.5 (2/4)	1.0 (5/5)	1.5 (6/4)
Pulse Amplitude (ng/ml)	-	2.65±0.36	4.57±0.51*
Nadir (ng/ml)	-	0.81±0.20	0.78±0.23

(* $p < 0.05$ compared with the inhibin immunised group,analysis of variance)

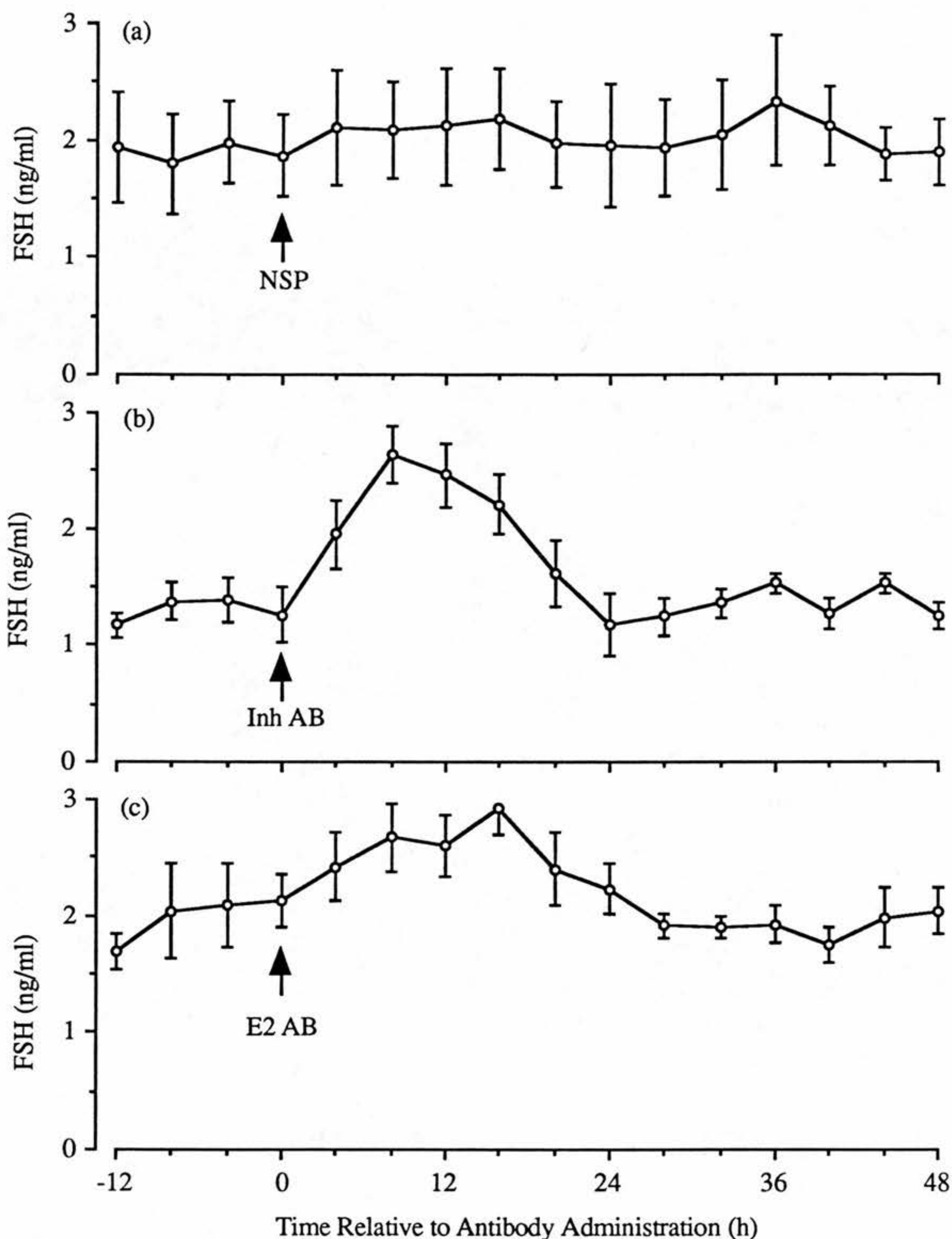

Fig. 9.3.
Mean (±S.E.M.) peripheral plasma concentrations of FSH in groups of ewes injected at time 0h on day 10 of the luteal phase with either (a) 10ml normal sheep plasma (NSP; n = 4), 10ml inhibin antibody (Inh AB; n = 5) or (c) 10ml oestradiol antibody (E2 AB; n = 4).

LH pulse amplitude or nadir in this group, however estimates were possible in the two immunised groups. The mean pulse amplitude was significantly ($p < 0.05$) higher in the oestradiol immunised than in the inhibin immunised group, while the nadir was similar in the two groups. While pulse frequency was highest in the oestradiol immunised group this difference was not significant.

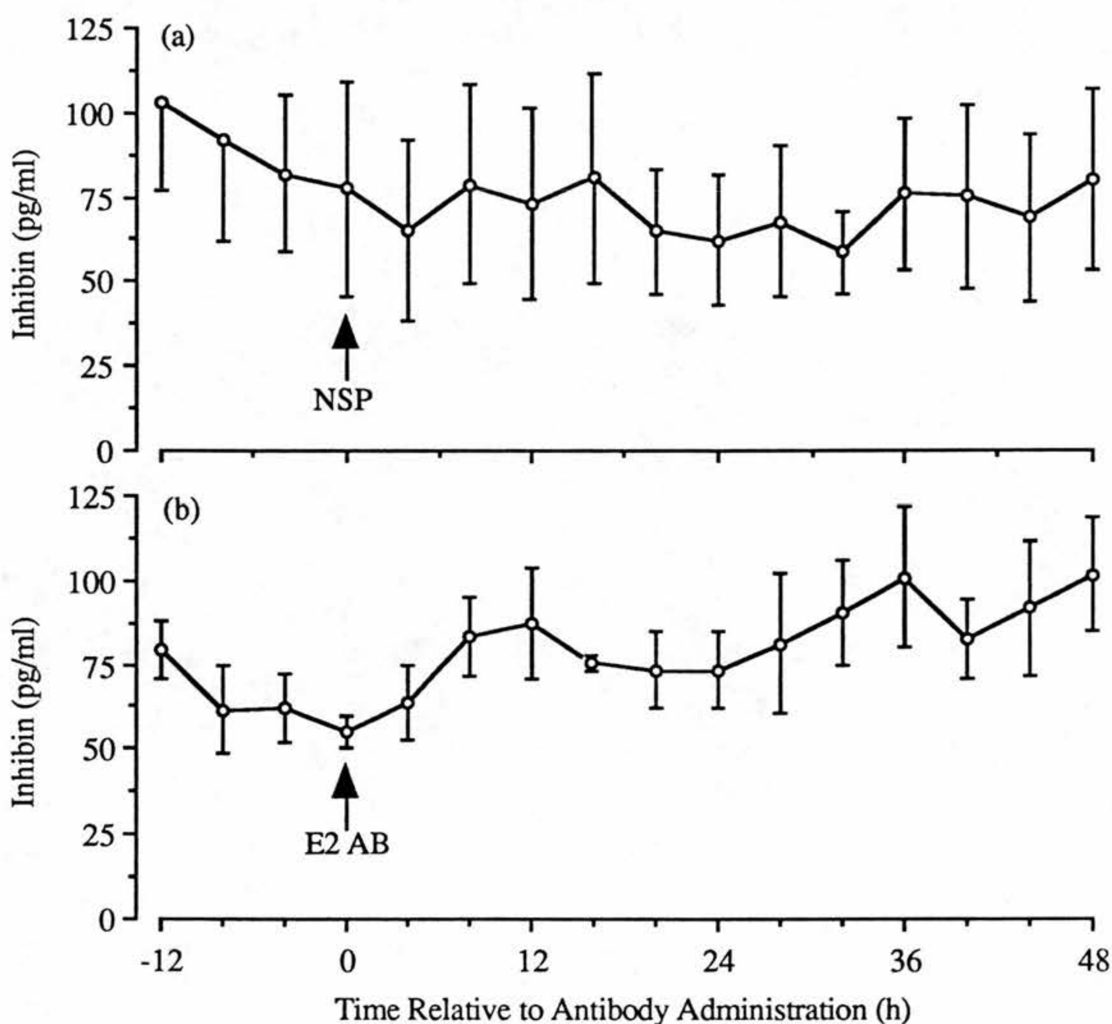

Fig. 9.4.
Mean (±S.E.M.) peripheral plasma concentrations of inhibin in groups of ewes injected at time 0h on day 10 of the luteal phase with either (a) 10ml normal sheep plasma (NSP; n = 4) or (b) 10ml oestradiol antibody (E2 AB; n = 4)

9.3.4. Ovarian Hormone Secretion Rates

The mean secretion rates of oestradiol, inhibin and androstenedione over the period of intensive blood sampling, from 12 to 20h following treatment are shown in Table 9.2. Due to the presence of antibodies to the hormones, inhibin was not measured in the inhibin immunised group and oestradiol was not measured in the oestradiol immunised group. The rate of oestradiol secretion in the inhibin immunised group was higher than in the control group, this difference approaching statistical significance ($p < 0.07$). The secretion rate of oestradiol in relation to peripheral LH concentration is shown in a representative animal from both the control group and the inhibin immunised group in Fig 9.5. The animal immunised against inhibin showed a much larger release of oestradiol in response to the pulse of LH than the control animal. While the mean secretion rate of inhibin over this period was higher in the oestradiol immunised group than in the control group this difference did not prove to be significant. The ovarian secretion rate of inhibin in the group of ewes immunised against oestradiol showed a somewhat variable but significant ($p < 0.05$) rise following antibody injection (Fig. 9.6), while the control group showed no significant change. This rise in inhibin secretion rate was significantly correlated ($R = 0.69$; $p < 0.05$) with the peripheral concentration of FSH during the first 16h period following immunisation (Fig. 9.7.). The mean ovarian secretion rates of androstenedione in the two immunised groups was considerably higher than in the control group, though these differences were not statistically significant.

Table 9.2.

Mean (±S.E.M.) rate of ovarian secretion of oestradiol, inhibin and androstenedione during the period of intensive blood sampling from 12 to 20h following injection of control plasma (n = 3), inhibin antibody (n = 4) or oestradiol antibody (n = 4) on day 10 of the luteal phase of the oestrous cycle.

	Control	Inhibin Immunised	Oestradiol Immunised
Oestradiol (ng/min)	0.42±0.10	0.87±0.17*	-
Inhibin (ng/min)	2.16±0.86	-	3.35±0.05
Androstenedione (ng/min)	3.03±1.11	5.98±1.21	7.76±2.91

(* $p < 0.07$, student's unpaired t - test)

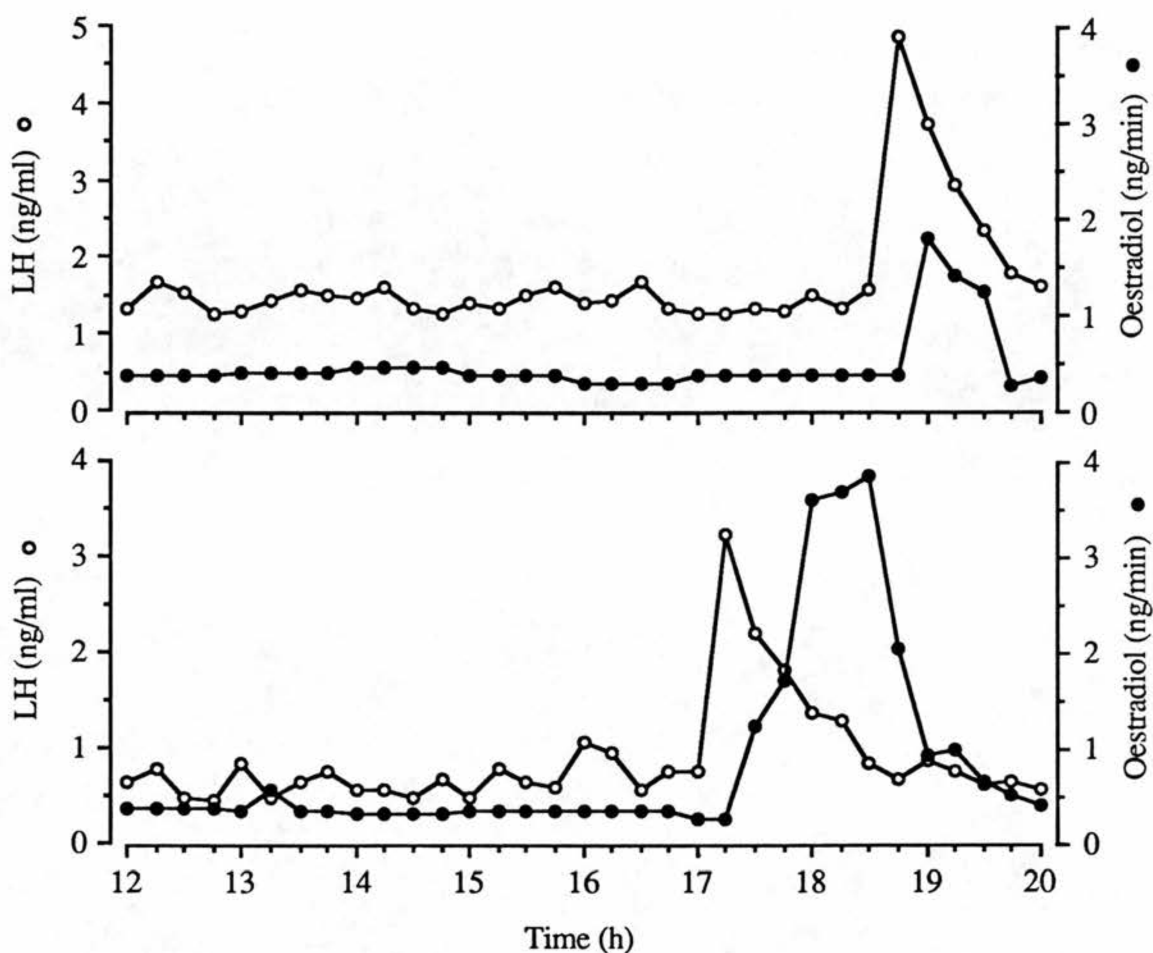

Fig. 9.5
Graph showing peripheral concentration of LH (o) and ovarian secretion rate of oestradiol (•) during the period of intensive blood sampling from 12 to 20h following treatment in a representative animal selected from (a) the control group and (b) the inhibin - immunised group. The ewes selected showed mean secretion rates of oestradiol over the period shown which were closest to the group means.

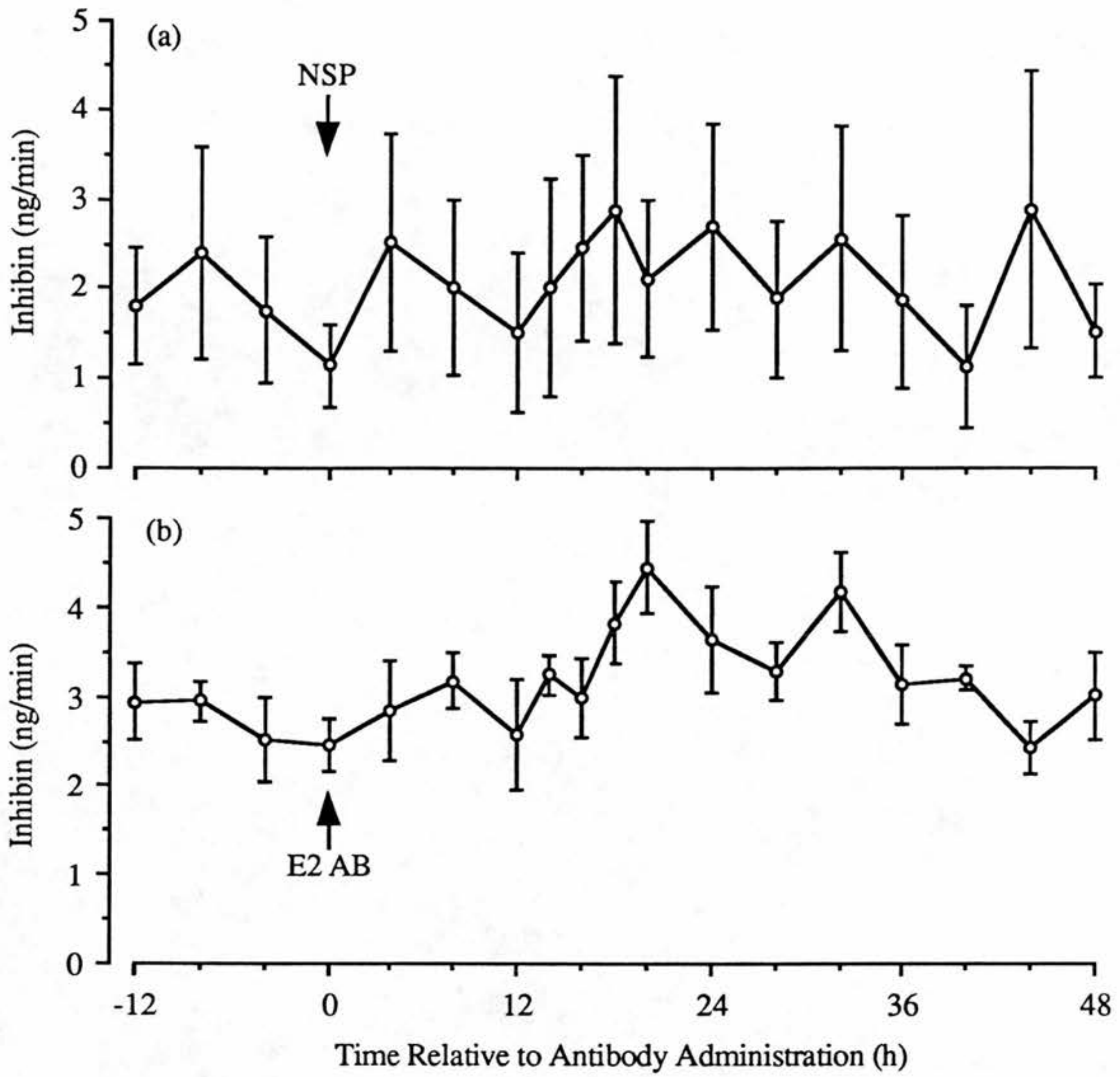

Fig. 9.6.
Mean (±S.E.M.) ovarian secretion rate of inhibin in ewes injected on day 10 of the luteal phase at time 0h with either (a) 10ml normal sheep plasma (NSP; n = 3) or 10ml oestradiol antibody (E2 AB; n = 4).

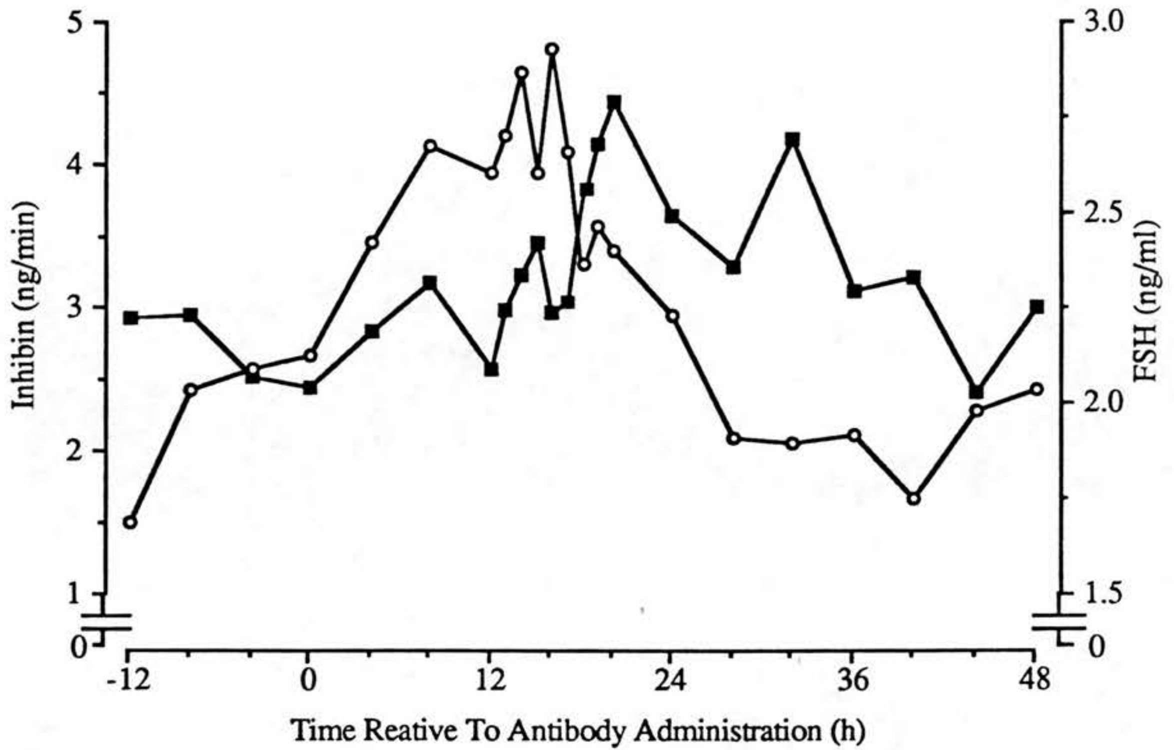

Fig. 9.7.
Mean peripheral concentration of FSH (○) and ovarian secretion rate of inhibin (■) in ewes passively immunised against oestradiol at time 0h on day 10 of the luteal phase of the oestrous cycle (n = 4).

9.4. Discussion

The rise in FSH concentration following immunisation resulted in a marked stimulation of follicle growth within 12h. The small rise in the number of follicles in the control group was probably, at least in part, an experimental artifact resulting from an increased familiarity with the ovaries as progressive scans were performed. Waves of follicle growth and regression occur throughout the oestrous cycle (Smeaton & Robinson, 1971; Mattner & Braden; 1972), and it is also possible that the rise in follicle number in the control group could have resulted from some animals in this group being in the rise phase of one of these waves. The rises in follicle number in the two immunisation groups were, however, significantly ($p < 0.001$) greater than in the control group demonstrating them to be a real effect of treatment. In the inhibin immunised animals there was a rise in the number of both small and medium sized follicles. This suggests that the rise in FSH had stimulated a new cohort of small

follicle with diameters below the sensitivity of the scanning procedure (< 2mm) to develop to sizes of up to 3.5 mm, as well as stimulating some of the follicles of up to 3.5 mm diameter present at the start of the experiment to develop to a diameter of 3.5 to 4.5 mm. In the oestradiol immunised group an increase in the number of small follicles was seen, but there was no increase in the number of medium sized follicles, and at 24h following treatment there were no large follicles present in this group. In this group the mean LH pulse amplitude was significantly ($p < 0.05$) higher than in the inhibin immunised group. This finding suggests that these high amplitude LH pulses had inhibited follicle development above a diameter of 3.5mm, a finding in keeping with Picton (1989), who also found that high amplitude LH pulses inhibited follicle development in FSH - stimulated GnRH suppressed ewes.

In both immunised groups there was a significant, though transitory rise in the concentration of FSH following treatment, this rise in FSH concentration lasting approximately 24h in both groups. This increase in FSH concentration was significantly ($p < 0.05$) larger in the inhibin immunised group than in the oestradiol immunised group, this fact possibly reflecting the low concentrations of oestradiol found during the luteal phase of the sheep oestrous cycle (Baird & McNeilly, 1981). In both groups the compensatory fall in FSH concentration was occurring during the period of rapid blood sampling from 12 to 20h following treatment, and so if changes in ovarian hormone secretion were involved in this compensatory mechanism they should be in evidence at this time. FSH has been shown to stimulate inhibin secretion (McNeilly et al., 1989), and in the group immunised against oestradiol there was a significant rise in the secretion rate of inhibin at this time of elevated FSH concentration. In the sheep, small follicles have been shown to produce significant amounts of inhibin (Chapter 4), and so this rise in inhibin secretion was probably partly due to the large number of small follicles stimulated to develop in this group. Inhibin secretion by granulosa cells has been shown to be responsive to stimulation by FSH (Bicksak et al., 1986; Suzuki, et al., 1987), and so this rise in inhibin secretion may also have resulted from an increase in inhibin secretion from those larger follicles already present in the ovary, and an increase in secretion from these larger follicles might have been responsible for the increase in peripheral inhibin concentration seen in the oestradiol immunised group immediately after treatment. The elevated secretion rate of inhibin, at the time when FSH levels were declining, provides evidence that an increase in the secretion rate of inhibin is involved in the compensatory fall in FSH seen following the post - oestradiol immunisation rise. It seems likely that the FSH - stimulated increase in follicle development results in an increase in follicular inhibin secretion which in turn exerts negative feedback on the pituitary resulting in the decline

seen in the secretion of FSH. Due to the acutely pulsatile nature of oestradiol release, and the infrequency of LH pulses during the luteal phase it is hard to demonstrate a rise in oestradiol secretion at this time. However, in the animal immunised against inhibin the mean secretion rate of oestradiol over the period of intensive blood sampling from 12 to 20h was higher than in the control group, though this differences was only marginally significant ($p < 0.07$). This finding does, however, suggest that there was an increase in the secretion rate of oestradiol during the period of compensation in FSH concentration following inhibin immunisation. As well as increases in inhibin secretion in response to oestradiol immunisation and oestradiol secretion in response to inhibin immunisation it is probable that immunisation against each hormone results in an increase in its own secretion rate. This idea is supported by the fact that active immunisation against androstenedione results in a large increase in the rate of secretion of the hormone (Campbell et al., 1990c). The mean secretion rates of androstenedione during the period of intensive blood sampling from 12 to 20h following treatment in the two immunised groups were approximately double that seen in the control group. While this difference was not significant, the mean androstenedione secretion rate in the two immunised groups in this study were also double the luteal phase secretion rate found in another study performed on these animals during the previous year (Campbell et al., 1990a). This suggests that the increase in the number of follicles > 2mm following passive immunisation against inhibin or oestradiol does result in an increase in the ovarian secretion rate of androstenedione.

The results of this study demonstrate that the elevation in the concentration of FSH resulting from passive immunisation against inhibin or oestradiol results in a large increase in the number of follicles > 2mm per ovary. The results also show that inhibin secretion rises in response to oestradiol immunisation, and that oestradiol secretion tends to rise following inhibin immunisation in order to maintain normal plasma FSH concentrations. This finding provides further evidence for the theory that inhibin and oeatradiol interact in the control of FSH secretion in the sheep.

Chapter 10

General Discussion

10.1. The Source of Ovarian Inhibin Production

The aim of the experiments carried out in Chapters 3 was to determine the ovarian source of inhibin in the ewe and in particular to try and clarify the ambiguity that exists over the role of the corpus luteum in inhibin production in the sheep. The ovarian follicles are now widely accepted as a major source of inhibin production in the sheep, and in this study the secretion of inhibin was found to be significantly higher from ovaries with large (≥ 3mm) antral follicle than from contralateral ovaries with only small (< 3mm) follicles. In this study the secretion rate of inhibin into the ovarian vein was found to be unaffected by the presence or absence of a corpus luteum in the ovary. Following enucleation of the corpora lutea the expected marked fall in the secretion rate of progesterone was seen, while inhibin secretion showed a much smaller fall, of similar magnitude to that seen in the secretion rate of oestradiol which is only produced by the follicles. This fall in the secretion rate of inhibin and oestradiol could be accounted for by the trauma of enucleation and the effects of anaesthesia and so these finding demonstrate that in the sheep, the corpus luteum does not secrete inhibin. In an earlier study Tsonis et al. (1988a) demonstrated that inhibin secretion was higher from ovaries containing luteal tissue and that this secretion declined following luteal regression, suggesting that, as in the rat and human, the corpus luteum of the sheep secretes inhibin. However, in this study samples were collected from both control ewes and ewes which had been immunised against androstenedione. As such immunisation increases both ovulation rate and the secretion rate of inhibin (Campbell et al., 1988), an over - representation of immunised animals in the samples from ovaries with corpora lutea presumably affected the results in this study. This lack of inhibin production by luteal tissue is supported by two studies in which attempts to demonstrate mRNA for inhibin in ovine and bovine luteal tissue were unsuccessful (Rodgers et al., 1989; Torney et al., 1989), and by Henderson & Franchimont (1981) who failed to show inhibin production from cultured bovine luteal cells.

While it now appears that the follicles are the only major source of ovarian inhibin production, there is a lack of knowledge on inhibin production from follicles at different stages of development. In Chapter 4 inhibin production was measured in individual follicles collected at various stages of the oestrous cycle. In agreement with other studies inhibin production was found to increase with increasing follicle diameter

and to be higher in oestrogenic than in non oestrogenic follicles. However, compared to oestradiol, which is produced almost exclusively by the largest one or two healthy antral follicles, a larger proportion of inhibin was produced by smaller antral follicles and by large non - oestrogenic atretic follicles, which together accounted for almost half of the total inhibin production. When oestradiol production from follicles was examined separately at different stages of the oestrous cycle more oestrogenic follicles were found during the early and mid stages of the follicular phase than during the late follicular phase or the luteal phase. In contrast, the number of follicles with high inhibin production was similar at all stages of the cycle. It appears, therefore, that while the level of oestradiol secretion by the ovary reflects the development of large oestrogenic follicles, the amount of inhibin the ovary secretes is more closely related to the total population of large antral follicles. This finding provides an explanation for the fact that inhibin shows less marked fluctuations during the oestrous cycle than oestradiol.

10.2. The Physiological Role of Inhibin

Inhibin is defined as a selective suppressor of FSH secretion, though in a number of studies high doses of inhibin have been shown to affect the secretion of LH in the sheep. In Chapter 5 a dose of inhibin approximately equivalent to normal ovarian output was shown to suppress FSH secretion in oestradiol benzoate - treated ovariectomised ewes while having no effect on the degree of LH suppression or on the characteristics of the LH surge which was induced by the oestradiol treatment. In the experiments described in Chapters 6 - 9 passive immunisation against inhibin had no effect on the peripheral concentration of LH during either the luteal or the follicular phase of the oestrous cycle. Furthermore, in the experiment described in Chapter 8, treatment with a dose of inhibin approximately equivalent to normal ovarian output had no effect on the rise in the concentration of LH seen following acute ovariectomy. These results support the idea that in studies where inhibin has been shown to have an effect on LH secretion, this was a pharmacological effect resulting from the use of amounts of inhibin far in excess of those seen in physiological situations. It would seem from the results presented in this study, therefore, that inhibin plays little if any role in the physiological control of LH secretion in the ewe.

The fact that passive immunisation against inhibin results in a marked rise in FSH secretion during both the luteal and the follicular phases of the oestrous cycle provides strong evidence that inhibin does play an important physiological role in the control of FSH secretion in the ewe. In Chapter 8, it was shown that doses of inhibin or oestradiol, estimated as approximately equivalent to normal ovarian output, were

each able to partially prevent the post - castration rise in FSH concentration in the ewe, while a combination of the two treatments was sufficient to completely prevent this rise. This result confirms the similar findings of Martin et al. (1988), and suggests that inhibin and oestradiol act together in the control of FSH secretion. This interaction of inhibin and oestradiol is further supported by the observation that passive immunisation against either hormone results in a moderate rise in the peripheral concentration of FSH, while a combined immunisation against both hormones results in a significantly larger increase in FSH concentration. In Chapter 8 immunisation against inhibin and oestradiol in combination was shown to cause a rise in the peripheral concentration of FSH similar to that seen following acute ovariectomy. This finding adds weight to the idea that inhibin and oestradiol act together in the control of FSH secretion, and suggests that inhibin and oestradiol are the only two hormones with an important role in the control of FSH secretion in the ewe.

Following luteal regression, during the early to mid follicular phase of the sheep oestrous cycle, there is an increase in the ovarian secretion rate of oestradiol, resulting from an increase in the production of oestradiol by the preovulatory follicle(s). This rise in oestradiol secretion is thought to be important in causing the decline seen in FSH concentration at this time which is, in turn, thought to be responsible for regulating the number of preovulatory follicles which develop to ovulation. In Chapter 3 the ovarian secretion rate of inhibin showed little change during this period while the secretion rate of oestradiol was significantly elevated. This finding, combined with the lack of variation in the pattern of inhibin production by individual follicles at different stages of the oestrous cycle and the relatively high level of inhibin production by large non - oestrogenic follicles shown in Chapter 4, suggests that inhibin does not play a major role in causing this decline in the secretion of FSH.

Active immunisation against oestradiol results in an increase in the peripheral concentration of FSH, and an increase in follicular development, though ovulation does not occur due to failure of oestradiol to induce a preovulatory LH surge (Martenz et al., 1979). In a number of studies, active immunisation of ewes against inhibin has been shown to cause an increase in ovulation rate. However, in these studies the expected increase in FSH concentration has been small and variable, or not detected. The reason for this lack of a detectable increase in the peripheral concentration in FSH is not known, but could result from a compensatory increase in the secretion of inhibitory substances by the ovary necessitating more acute monitoring of FSH concentrations. The passive immunisation studies reported in this thesis show that immunisation against inhibin does result in an increase in the peripheral concentration of FSH, but that this rise is transitory. This suggests that some form of compensation does occur to maintain

normal concentrations of FSH. In Chapter 9 this compensatory mechanism was investigated. Immunisation against inhibin or oestradiol resulted in a large increase in the number of follicles per ovary. In the animals immunised against inhibin there was an increase in the secretion rate of oestradiol, while in the animals immunised oestradiol there was an increase in the secretion rate of inhibin. These finding suggest that inhibin and oestradiol are, to some extent, interchangeable in the control of FSH secretion, and suggest that the two hormones are acting together to regulate the peripheral concentration of FSH.

10.3. Conclusions

From the data presented in this thesis it can be concluded that, in the sheep, the main source of inhibin is the large antral follicles, and that the corpus luteum secretes little, if any, immunoactive or bioactive inhibin. Furthermore, the results demonstrate that compared to their small contribution to total oestradiol production, small antral and large non - oestrogenic follicles produce large quantities of inhibin, this more even distribution of follicle inhibin production being reflected in relatively small variations in ovarian inhibin secretion during the oestrous cycle. The results of this study also suggest that inhibin has no major role in the physiological control of LH secretion in the ewe, supporting the definition of inhibin as a selective suppressor of FSH. The passive immunisation studies show that inhibin has an important role in the control of FSH secretion, and together, the results of these studies demonstrate that inhibin and oestradiol act together in the control of FSH secretion in the ewe. It seems most probable that the changes in FSH concentration occurring during the oestrous cycle, which control follicle selection and ovulation rate, result mainly from changes in the secretion of oestradiol, while inhibin, with its much longer half life, combines with oestradiol to set the overall level of negative feedback.

Chapter 11

Appendix

11.1. Assay Protocols

11.1.1. Assay Buffers

0.075 Molar Phosphate Buffered Saline (PBS)

10.013 g $Na_2HPO_4.2H_2O$

2.925 g $NaH_2PO4.2H_2O$

4.38 g NaCl

0.1 g thiomersalate

1 l deionised water

Phosphate Citrate Buffer (PCB)

17.85 g $Na_2HPO_4.2H_2O$

7.75 g citric Acid

0.1 g thiomersalate

1 l deionised water

Phosphate Gelatin Bufered Saline (PGBS)

10.86 g $Na_2HPO_4.2H_2O$

6.08 g $NaH_2PO4.2H_2O$

9.0 g NaCl

0.1 g thiomersalate

1.0 g gelatin

1 l deionised water

11.1.2. Progesterone Assay

Buffer - PCB + 0.1% gelatin (PCBG), pH 6.0.

Day 1 - 50 µl sample + 100 µl buffer

or 100 µl standard + 50 µl ovx plasma

100 µl antibody (Anti Progesterone 361 at 1:10000)

100 µl ^{125}I tracer (15000 cpm in PCB + 1mg/ml ANS)

Incubate overnight at 4°C

Day 2 - 1ml 0.9% saline + 15% PEG then spin at 2000g for 30 min at 4°C

Aspirate supernatant and count precipitate.

11.1.3. M91/1 FSH Assay

Buffer - PBS + 1.0% BSA, pH 7.4

Day 1 - 100 μl sample/standard

300 μl buffer

100 μl antibody (M91/1; 1:5000 in special buffer - PBS +0.6% NRS)

Incubate for 1 day at 4°C

Day 2 - 100 μl ^{125}I tracer (15000 cpm)

Incubate for 1 day at 4°C

Day 3 - 100 μl DARS (1:16 in PBS + 4% dextran)

Incubate overnight at 4°C

Day 4 - 0.7 ml PBS + 5% PEG then spin at 2000g for 30 min at 4°C

Aspirate supernatant and count precipitate.

11.1.4. NIAMMD - I - 1 FSH Assay

Buffer - PBS + 1.0 % BSA, pH 7.4.

Day 1 - 150 μl sample/standard

150 μl buffer

50 μl antibody (NIAMMD - oFSH - I - 1 at 1:12000)

Incubate for 1 day at 4°C

Day 2 - 50 μl ^{125}I tracer (15000 cpm)

Incubate for 1 day at 4°C

Day 3 - 100 μl DARS (5257M 1:32)

100 μl NRS (5106L 1:800)

Incubate overnight at 4°C

Day 4 - 1ml 0.9% saline then spin at 2000g for 30 min at 4°C

Aspirate supernatant and count precipitate.

11.1.5. LH Assay

Buffer - PBS + 0.1% BSA, pH 7.4.

Day 1 - 100 µl sample/standard
200 µl buffer
100 µl antibody (Anti oLH R29 at 1:120000)
Incubate for 1 day at 4°C

Day 2 - 100 µl ^{125}I tracer (15000 cpm)
Incubate for 1 day at 4°C

Day 3 - 100 µl DARS (5257M 1:16; 5104L 1:32)
100 µl NRS (5106L 1:800)
Incubate overnight at 4°C

Day 4 - 1ml 0.9% saline then spin at 2000g for 30 min at 4°C
Aspirate supernatant and count precipitate.

11.1.6. Inhibin Assay

Buffer - PBS + 1.0% BSA, pH 7.5.

	Plasma	Medium
Day 1 -	200 µl sample + 100 µl buffer	50 µl sample/standard
	100 µl standard + 200 µl ovx plasma	250 µl buffer
	100 µl antibody (Anti 1 - 26α R150 at 1:15000)	
	Incubate for 1 day at 4°C	
Day 2 -	100 µl ^{125}I tracer (15000 cpm)	
	Incubate for 1 day at 4°C	
Day 3 -	100 µl DARS (5257M 1:40)	
	100 µl NRS (5162L 1:500)	
	Incubate overnight at 4°C	
Day 4 -	1ml 0.9% saline then spin at 2000g for 30 min at 4°C	
	Aspirate supernatant and count precipitate.	

11.1.7. Oestradiol Assay

Buffer - PBS + 0.1% gelatin (PBSG), pH 7.2.

Extraction - 100 μl sample/standard + 1ml diethyl ether (AnalR) to 75 x 12 glass tubes
Vortex for 2 x 30 seconds. Freeze in dry ice/ethanol bath
Decant ether extract into 75 x 10 mm glass tubes
Dry down in heating block (40°C) under gas (N_2)

	Plasma Extraction Assay	**Medium Assay**
Day 1 -	200 μl antibody (anti E_2 BW at 1:2000000)	100 μl sample/standard
	200 μl ^{125}I tracer (15000 cpm)	100 μl buffer (PGBS)
		100 μl antibody (1:1000000)
		100 μl ^{125}I tracer (15000 cpm)

Incubate overnight at 4°C

Day 2 - Place tubes on ice and add 500 μl PGBS + 0.125% dextran + 1.25% charcoal
Vortex and leave on ice for 10 min
Centrifuge at 2000g for 10 min at 4°C
Decant supernatant into LP3 plastic tubes and count

11.1.8. Androstenedione Assay

Buffer - PBS + 0.25% BSA, pH 7.4.

Extraction - 200 μl sample/standard + 2ml 4:1 hexane:diethyl ether to 75x12 glass tubes
Vortex for 2 x 60 seconds then freeze in dry ice/ethanol bath
Decant ether extract into 75 x 10 mm glass tubes
Dry down in heating block (40°C) under gas (N_2)

	Plasma Extraction Assay	**Medium Assay**
Day 1 -	200 μl antibody (anti A_4 at 1:56000)	100 μl sample + 100 μl buffer
	200 μl ^{125}I tracer (10000 cpm)	(PBS + 0.25% BSA)
		or 200 μl standard
		100 μl antibody (1:28000)
		100 μl ^{125}I tracer (10000 cpm)

Incubate overnight at 4°C

Day 2 - 200 μl DARS (5257M 1:40) + 200 μl NRS (5162L 1:500)

Incubate overnight at 4°C

Day 3 - Spin at 2000g for 45 min at 4°C and aspirate supernatant and count precipitate

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