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Relationship between LH receptor concentrations in thecal and granulosa cells and in-vivo and in-vitro steroid secretion by ovine follicles during the preovulatory period

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Summary. Ewes were ovariectomized before (Group 1, N = 5) or after (Group 2, N = 6) the peak of the preovulatory gonadotrophin surge. Ovarian secretion rates of oestradiol and testosterone were significantly higher in Group 1 than in Group 2. The presence of high levels of LH receptors in both thecal and granulosa cells was used to identify ovulatory from non-ovulatory follicles. There was a significant fall in the LH receptor concentration in the thecal and granulosa cells of ovulatory follicles after the peak of the preovulatory gonadotrophin surge. Ovulatory follicles in Group 1 produced significantly more oestradiol and testosterone *in vitro* than did those in Group 2. In both groups ovulatory follicles secreted significantly more oestradiol *in vitro* than did non-ovulatory follicles. Follicular fluid oestradiol concentrations were similar in pattern to the in-vitro oestradiol secretion activity in ovulatory and non-ovulatory follicles. However, follicular fluid testosterone concentrations were significantly higher in non-ovulatory follicles than in ovulatory follicles. Incubation of follicles with 250 ng testosterone/ml did not significantly alter the in-vitro oestradiol secretion rate in any of the groups of follicles except for Group 2 non-ovulatory follicles in which oestradiol accumulation increased. The number of thecal and granulosa cell LH receptors was significantly correlated with follicular fluid oestradiol concentrations in ovulatory follicles and with in-vitro oestradiol production by Group 1 ovulatory follicles. It is suggested that the fall in oestradiol secretion rates, which occurs after the peak of the preovulatory gonadotrophin surge, may be due to a decrease of aromatase activity associated with a fall in the concentration of LH receptors and is not due to a lack of the oestrogen precursor testosterone. The elevated concentration of testosterone and low oestradiol concentrations in non-ovulatory follicles compared with ovulatory follicles are probably due to an inactive aromatase system, perhaps associated with the lack of granulosa cell LH receptors.

Introduction

Previous studies of follicular development and steroidogenesis in the ewe have concentrated on the effects of follicular size, the presence of gonadotrophic hormones and stage of the oestrous cycle. It has been demonstrated that follicular size is positively correlated with follicular fluid

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steroid concentrations, but only during the preovulatory period of the sheep oestrous cycle (England, Dahmer & Webb, 1981a). Most of the oestradiol in the peripheral circulation, during both the luteal and follicular phases of the oestrous cycle in the ewe, is secreted by the ovary containing the largest Graafian follicle (Bjersing *et al.*, 1972; Baird & Scaramuzzi, 1976; McNatty, Gibb, Dobson, Thurley & Findlay, 1981; England, Webb & Dahmer, 1981b). Other studies have shown that the largest one or two ovarian follicles produce significantly more oestradiol *in vitro* than do smaller follicles taken from the same ewes (Moor, 1973). Furthermore, the capacity of follicles to secrete oestrogen *in vitro* increased when follicles were taken from sheep during the follicular phase of the oestrous cycle.

More recent studies (England *et al.*, 1981b; Webb & England, 1982) have demonstrated that ovulatory follicles, larger and containing more oestradiol than non-ovulatory follicles, can be identified by the presence of a significant number of LH receptors in both the granulosa and thecal cells. The ovary containing the ovulatory follicle secreted the increased concentrations of oestradiol found during the preovulatory period. Nevertheless, the intimate relationships between gonadotrophic hormones, gonadotrophin receptors and steroidogenesis in ovulatory and non-ovulatory follicles of the ewe are not known. This study was designed to investigate these relationships in both types of follicles during the preovulatory period as there is a rapid fall in peripheral oestradiol concentrations approximately 2 h before the peak of the gonadotrophin surge (Webb, England & Fitzpatrick, 1981). This provides two closely timed yet contrasting periods during the oestrous cycle of the ewe; one with high follicular steroid production and the other with significantly decreased steroid production.

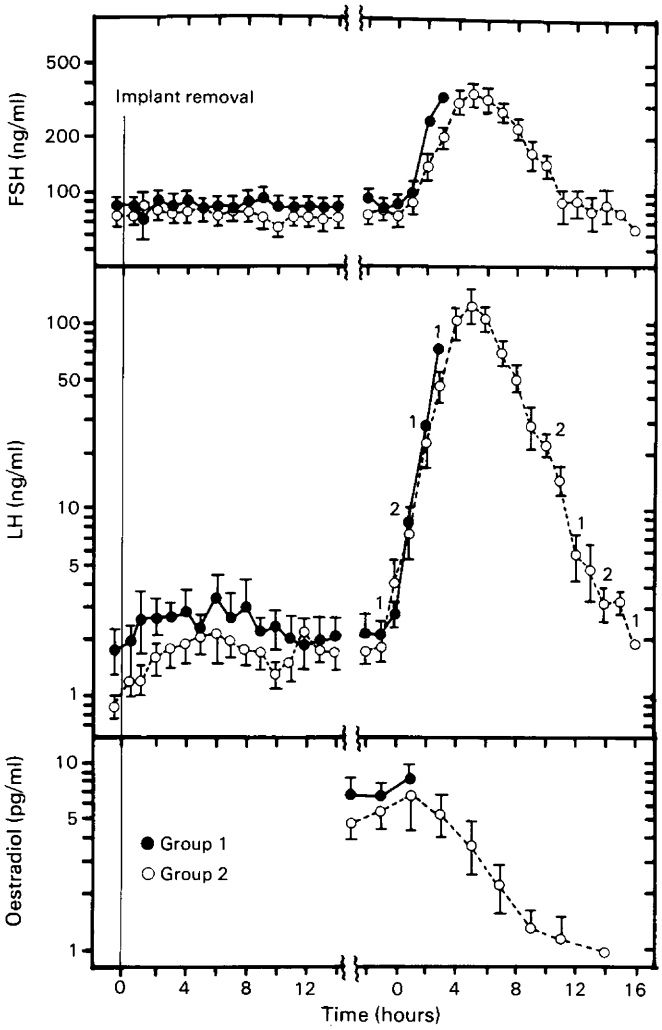
Materials and Methods

Animals and treatments

Oestrus was synchronized in 11 Suffolk ewes, as described by Hauger, Karsch & Foster (1977), by using Silastic implants containing progesterone (Sil-Estrus: Abbott Laboratories, Chicago, Illinois). Beginning from the time of implant removal, the ewes were penned with a raddled vasectomized ram, and bled via jugular venepuncture and checked for oestrus at hourly intervals. Follicles were obtained by ovariectomizing the ewes 2 or 10 h after the first signs of behavioural oestrus. Gonadotrophin concentrations (see Text-fig. 1) in the hourly blood samples were used retrospectively to determine that 5 ewes had been ovariectomized before (Group 1) and 6 ewes after (Group 2) the peak of the preovulatory gonadotrophin surge. Ovariectomy was performed through a mid-ventral incision while the ewes were under pentobarbitone sodium anaesthesia. The ovarian veins were cannulated immediately before ovariectomy and the rate of ovarian steroid secretion was calculated as described previously (England *et al.*, 1981b). Dissection of follicles $\geqslant$2 mm in diameter from adhering stromal tissue was completed within 1 h of ovariectomy. The dissected follicles were incubated individually in Medium 199 (Grand Island Biological Co., Grand Island, New York) for 4 h in a Dubnoff shaking water bath at 37°C in an atmosphere of 45% oxygen, 50% nitrogen and 5% carbon dioxide (Moor, 1973). The follicles were transferred into fresh M199 at 30 min intervals and 250 ng testosterone were added to the incubation media for the final 2 h of the incubation period. After incubation follicles were stored in liquid nitrogen until thecal and granulosa cells were separated and ^{125}I-labelled hCG binding analysis was performed (Webb & England, 1982). The culture medium was stored at −20°C until measurements of oestradiol and testosterone concentrations.

Validation of the incubation procedure

To determine whether the steroid concentrations in the culture media were the result of de-novo synthesis or passive diffusion from the follicular fluid, 6 ewes were ovariectomized and



Text-fig. 1. Mean (±s.e.m.) concentrations of LH, FSH and oestradiol in peripheral serum of sheep ovariectomised before (Group 1, N = 5) or after (Group 2, N = 6) the peak of the gonadotrophin surge. The numbers in the LH panel depict the number of sheep ovariectomized at the time indicated. The concentrations in these ovariectomized sheep are not included in the subsequent datum points. Values before the break in the horizontal axis are normalized from the time of implant removal and data after the break are normalized to the start of the preovulatory LH surge.

follicles dissected from the surrounding ovarian stroma as described previously (Webb & England, 1982). Follicles (n = 17; diam. 1·0–7·0 mm) from 3 ewes (Group A) were incubated for 90 min in Medium 199. The follicles were then ruptured, the follicular fluid removed and the tissue washed 3 times in Medium 199 followed by a further 90 min incubation period. Follicles (n = 21; diam. 2·5–7·0 mm) from 3 other ewes (Group B) were ruptured before incubation, the follicular fluid removed, and the tissue washed 3 times before incubation for 3 h. There was no significant fall in oestradiol accumulation if the follicular fluid was removed half way through the incubation period or before incubation. Oestradiol secretion during the first 90 min of incubation was assigned as 100% and mean (±s.e.m.) oestradiol secretion in the final 90 min of incubation

was 100·6 ± 10·0% for Group A and 121·0 ± 12·3% for Group B. Oestradiol secretion ranged from 4 to 1800 pg/ml/30 min in Group A and from 6 to 1041 pg/ml/30 min in Group B. This finding supports the conclusion of Moor (1973) that in-vitro oestradiol secretion is similar in broken and intact follicles and the values obtained from the incubation media are indicative of actual steroid secretion and not diffusion from the follicular fluid.

Hormone assays

Concentrations of LH in peripheral serum were determined in duplicate by a radioimmunoassay (Niswender, Reichert, Midgley & Nalbandov, 1969) modified as described by Hauger *et al.* (1977). LH concentrations are expressed in terms of NIH-LH-S12. Peripheral serum FSH concentrations were determined by the radioimmunoassay described by Goodman, Pickover & Karsch (1981). FSH concentrations, expressed in terms of NIH-FSH-S8, were determined in duplicate 60 μl serum samples. All samples were measured in one assay. Oestradiol concentrations in peripheral and ovarian venous serum were determined in duplicate using a previously described radioimmunoassay (England, Niswender & Midgley, 1974). All samples were subjected to extraction and Sephadex LH-20 chromatography to remove all other major cross-reacting substances (Hauger *et al.*, 1977). It was found that oestradiol could be analysed in culture media and follicular fluid without extraction (England *et al.*, 1981a; Richards & Kersey, 1979). Testosterone concentrations in ovarian venous serum and follicular fluid were determined after extraction using a previously described radioimmunoassay (Foster *et al.*, 1978; England *et al.*, 1981a). Testosterone concentrations in the culture media were measured using the assay as described above. In these samples extraction procedures were omitted since the values obtained from non-extracted samples did not differ from those of extracted samples. Assay statistics are listed in Table 1.

Table 1. Assay statistics

Assay	Sensitivity/ml	Median variance ratio (%)*	Inter-assay coefficient of variation (%)
LH	0·3 ng	2·4	4·6
FSH	23·3 ng	2·1	—
Oestradiol			
Extracted samples	1·0 pg	4·9	11·9
Non-extracted samples	5·6 pg	11·8	9·6
Testosterone			
Extracted samples	13·9 pg	5·4	9·3
Non-extracted samples	15·5 pg	6·2	7·8

* The median variance ratio is an index of intra-assay variation (Duddleson, Midgley & Niswender, 1972).

^{125}I-labelled hCG binding in vitro

LH receptor studies (Webb & England, 1982) were carried out on all follicles ⩾3 mm in diameter. All procedures were carried out on ice unless otherwise stated. Thecal and granulosa cells were separated using a modification of the techniques described by Moor (1977) and Carson, Richards & Kahn (1981). The follicles were opened up in 1 ml phosphate-buffered saline (PBS, 0·1 M, pH 7·4) using fine scissors. The inside of the follicles was scraped with a blunt-ended needle, 18–22 gauge depending on the size of the follicle. The thecal shells were then inverted up the inside of a Pasteur pipette to remove the remaining granulosa cells and finally transferred to fresh PBS for washing before homogenization in a Dounce glass homogenizer (20

strokes) with a further 1 ml PBS. To ensure that this method did adequately separate the thecal and granulosa cells, samples from 12 follicles were fixed in Bouin's fluid, dehydrated in a series of graded ethanols up to 100%, embedded in paraffin wax blocks and then sectioned at a thickness of 8 μm. The sections were stained with haematoxylin and eosin or Masson's trichrome. Microscopic examination indicated that there was no contamination in any of the thecal shells with granulosa cells (see Pl. 1, Figs. 1–3). This provides evidence that this method gives excellent separation of the thecal shell from the granulosa cells. The combining of the two procedures of Moor (1977) and Carson *et al.* (1981), each effective alone, ensured that there would be negligible contamination between the different cell types even though the method prevented histological examination of the follicular tissue after incubation and hCG binding studies.

After homogenization the separated thecal and granulosa cells were centrifuged at 30 000 ***g*** for 10 min to obtain a crude membrane preparation. The supernatant from the granulosa cell homogenate was removed and stored at −20°C for steroid analysis as this contained the follicular fluid. This procedure may also contaminate the follicular fluid with granulosa cell cytoplasm, but this contamination seems to be minimal as follicular fluid oestradiol concentrations in this study were similar to those in follicles removed at a similar stage of the oestrous cycle in previous studies (England *et al.*, 1981b; McNatty *et al.*, 1981) in which follicular fluid was not contaminated with granulosa cell cytoplasm. The follicular fluid volume was calculated using an algorithm (England *et al.*, 1981a) which used the measured follicular diameter. Steroid levels in the follicular fluid could therefore be expressed as a concentration. The thecal and granulosa cell homogenates were washed for a second time, resuspended and then incubated with approximately 38 fmol ^{125}I-labelled hCG (Roussel, New York; 11 500 i.u./mg) with or without a 200-fold excess of unlabelled hormone (Roussel; 2950 i.u./mg). All samples were assayed in duplicate and incubated in a total volume of 120 μl for 16 h at 25°C. The reaction was terminated by adding 1 ml cold (4°C) PBS, containing 0·5% silica gel and 0·2% bovine serum alubmin. The mixture was centrifuged at 30 000 ***g*** for 10 min, the supernatant was decanted and the pellet resuspended and recentrifuged. The resulting supernatant was discarded and the pellet counted in a Searle 1185 gamma counter. Specific binding of the radiolabel to thecal or granulosa cell receptors was expressed as fmol ^{125}I-labelled hCG bound per μg DNA. DNA was determined by the method of Burton (1956). The effect of freezing follicular tissue in liquid nitrogen was determined by comparing LH receptor values in 4 Suffolk ewes, ovariectomized 1·5 ± 0·5 h after the start of the preovulatory gonadotrophin surge, with LH receptor levels in Group 1 sheep which were ovariectomized 1·6 ± 0·7 h after the start of the preovulatory gonadotrophin surge. Ovaries from the ewes were cooled on ice, the follicles were immediately removed from the surrounding ovarian stroma, and analysed for ^{125}I-labelled hCG binding. The concentration of LH receptors in theca and granulosa cells did not differ significantly between the two groups of ewes: the binding was 3·1 ± 0·7 ($n = 4$) and 2·4 ± 0·7 ($n = 9$) fmol ^{125}I-labelled hCG/μg DNA for fresh and frozen granulosa cell tissue respectively and 4·0 ± 1·0 ($n = 4$) and 2·7 ± 1·2 ($n = 5$) fmol/μg DNA for thecal cell tissues. A preparation of rat ovarian tissue, with a capacity of 4·2 ± 0·2 fmol ^{125}I-labelled hCG/μg DNA ($n = 6$) was included in all of the assays to assess interassay variability (11·5%, $n = 6$). The active fraction, a measure of the percentage of labelled hormone capable of binding to receptor, was 56·0 ± 1·2% ($n = 9$). The specific activity, estimated as described previously (Diekman, O'Callaghan, Nett & Niswender, 1978; Webb & England, 1982) was 27·8 ± 3·4 μCi/μg ($n = 9$).

Statistical analyses

Unless otherwise stated, all statistical analyses were carried out using a one-way analysis of variance (ANOV) incorporating Scheffe's test (1959). This method of analysis allows the use of

non-orthogonal ANOV comparisons. When the data (see Text-figs 2 and 3) were not normally distributed they were logarithmically transformed before statistical analysis. Transformed data were decoded after analysis and are presented as geometric means. All other means are presented as arithmetric means together with standard error of the mean (s.e.m.).

Results

Peripheral hormone concentrations

The pattern of peripheral LH, FSH and oestradiol concentrations in serum (Text-fig. 1) was similar for ewes in Groups 1 and 2. By approximately 4 h after removal of the progesterone implant serum LH concentrations had increased significantly in Groups 1 and 2 ($P < 0.05$; Friedman two-way analysis of variance). Serum FSH levels remained constant until the onset of the preovulatory gonadotrophin surge when peripheral concentrations increased dramatically. Oestradiol levels reached peak concentrations approximately 2 h before the peak of the gonadotrophin surge and within 8 h had decreased to approximately 1 pg/ml (Text-fig. 1). There was no difference in the interval from progesterone implant removal until the first signs of behavioural oestrus between Groups 1 (30.4 ± 1.7 h; $n = 5$) and 2 (26.5 ± 1.2 h; $n = 6$). Ovariectomy was carried out 1.6 ± 0.7 h after the start of the preovulatory gonadotrophin surge for Group-1 ewes and 9.7 ± 2.5 h after the peak of the preovulatory surge for Group-2 ewes.

^{125}I-labelled hCG binding to thecal and granulosa cells

Follicles were designated as ovulatory or non-ovulatory based on the concentrations of LH receptors in the granulosa cells. As described previously (Webb & England, 1982) there was a bimodal distribution of LH receptor capacity; follicles in Group 1 with >0·7 fmol ^{125}I-labelled hCG/μg DNA bound to the membrana granulosa were characterized as ovulatory, and non-ovulatory follicles had <0·2 fmol/μg DNA (Text-fig. 2a). Binding of ^{125}I-labelled hCG to thecal and granulosa cells of ovulatory follicles decreased significantly after the peak of the preovulatory gonadotrophin surge ($P < 0.05$). Non-ovulatory follicles had more LH receptors present in the thecal cells than in the granulosa cells, although the concentration was significantly less than in thecal cells of ovulatory follicles in both groups ($P < 0.05$). LH receptor concentrations in thecal and granulosa cells of non-ovulatory follicles decreased after the peak of the preovulatory gonadotrophin surge. The concentration of LH receptors in thecal cells was significantly correlated with LH receptor concentration in granulosa cells in Group 1 ($r = 0.72$; $n = 17$; $P < 0.01$) and Group 2 ($r = 0.87$; $n = 18$; $P < 0.01$).

Follicular fluid steroid concentrations

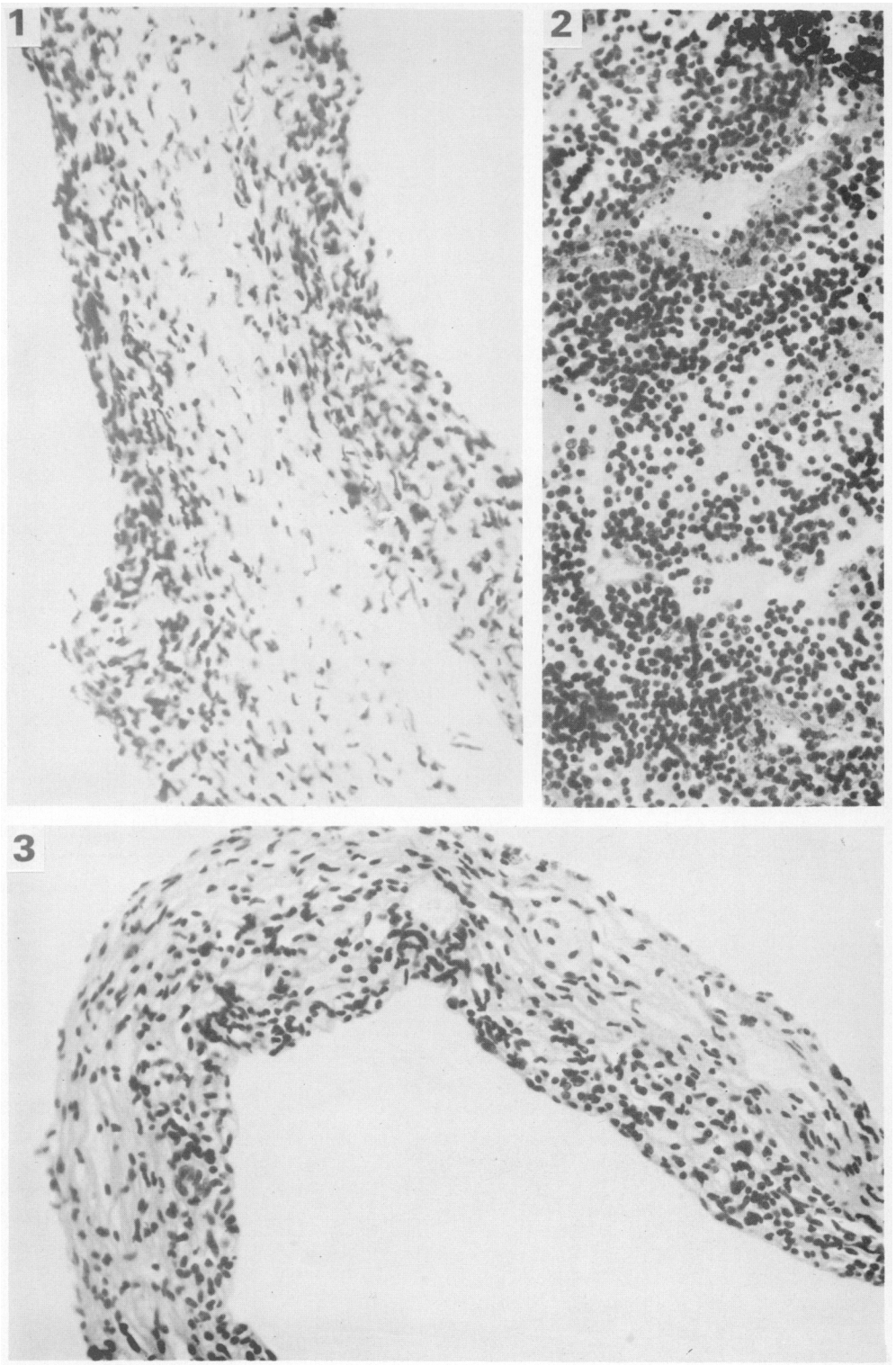
Follicular fluid oestradiol concentrations in ovulatory follicles were significantly higher in Group 1 than in Group 2 ($P < 0.05$) and oestradiol concentrations were significantly higher in ovulatory than in non-ovulatory follicles ($P < 0.05$) (Text-fig. 2b). In contrast, follicular fluid testosterone concentrations were significantly higher ($P < 0.05$) in non-ovulatory than in ovulatory follicles. There was a significant correlation between the concentrations of oestradiol in the follicular fluid and LH receptors in the thecal cells ($r = 0.69$; $P < 0.01$; $n = 14$) and granulosa cells ($r = 0.67$; $P < 0.01$; $n = 14$) of ovulatory follicles.

PLATE 1

Figs 1 and 3. Isolated thecal shells from sheep antral follicles. H & E, ×125.

Fig. 2. Isolated granulosa cells from a sheep antral follicle. H & E, ×125.

PLATE 1



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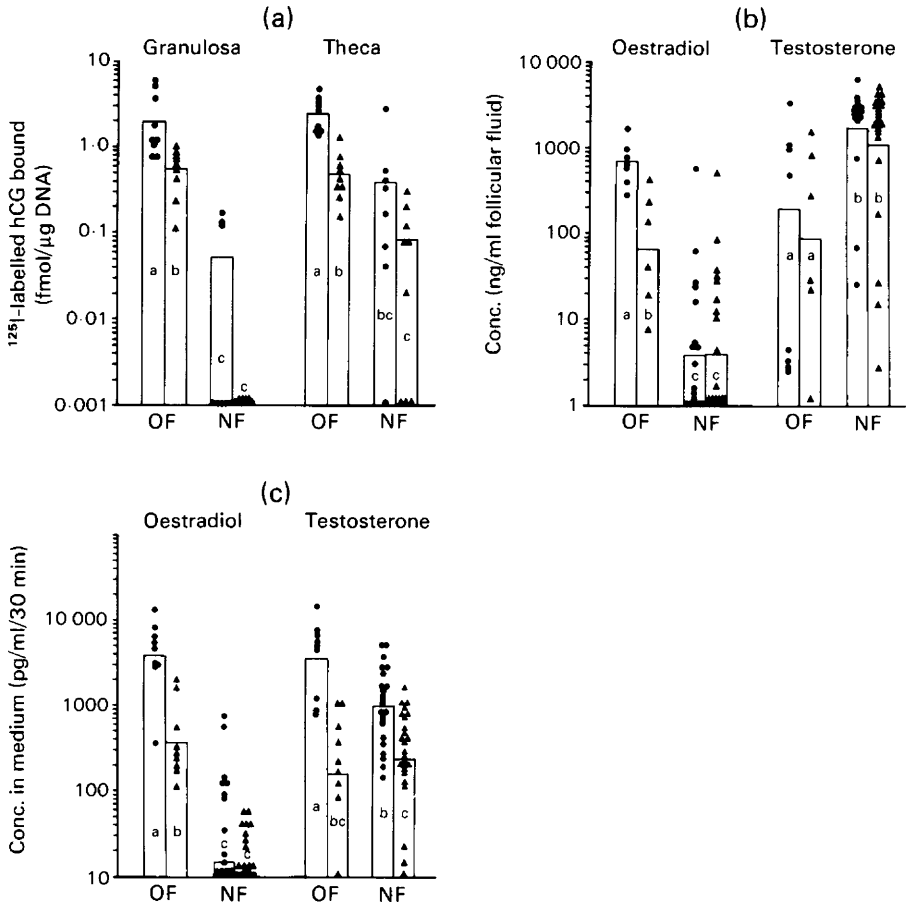


Text-fig. 2. Geometric mean concentrations of (a) ^{125}I-labelled hCG bound to thecal and granulosa cells, (b) follicular fluid concentrations of oestradiol and testosterone and (c) in-vitro accumulation of oestradiol and testosterone in culture media during the first 2 h of incubation by ovulatory (OF) and non-ovulatory (NF) follicles of ewes in Group 1 (●) and Group 2 (▲). Bars bearing different letters (a, b or c) are significantly different ($P < 0{\cdot}05$). Because the data have been expressed as a geometric mean, individual values have been presented.

In-vivo and in-vitro secretion of testosterone and oestradiol

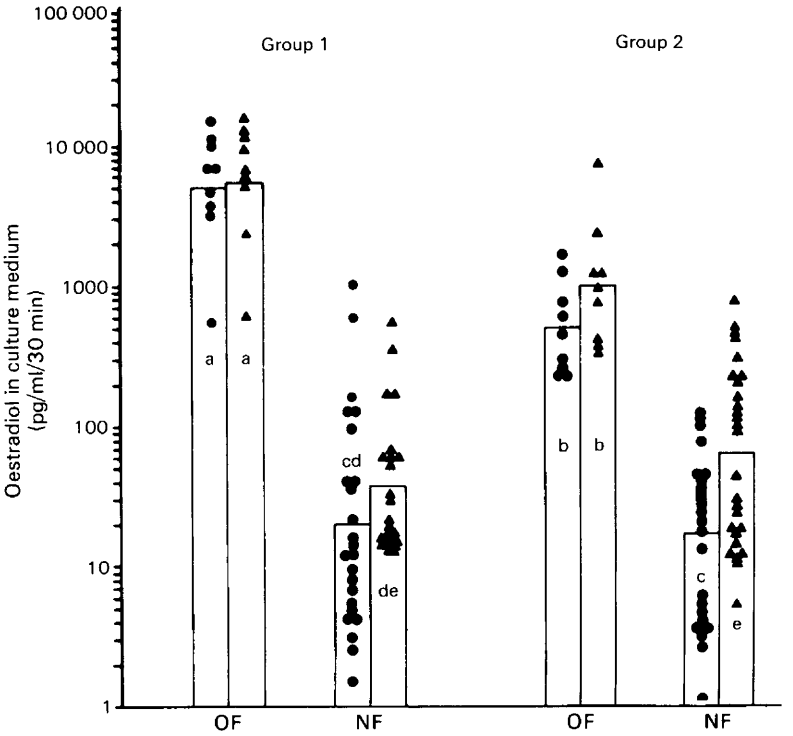
Ovarian secretion of testosterone and oestradiol was much lower in Group 2 than in Group 1 (Table 2). The accumulation of oestradiol in the culture media from the ovulatory follicles of Group 1 was significantly greater than those of Group 2 ($P < 0{\cdot}05$) (Text-fig. 2c). Also

Table 2. In-vivo ovarian secretion rates† of testosterone and oestradiol

	Group 1 ewes	Group 2 ewes
Testosterone (ng/min)	*10·1 ± 1·3 (4)	2·7 ± 0·7 (4)
Oestradiol (ng/min)	*6·4 ± 1·3 (5)	0·7 ± 0·2 (4)

† Secretion rates from both ovaries are added to give a total ovarian secretion rate per ewe. Values are mean ± s.e.m. for the no. of ewes indicated in parentheses.

* Significantly different from value for Group 2 ewes ($P < 0{\cdot}05$; Mann-Whitney Rank Sum Test).



Text-fig. 3. Geometric mean in-vitro accumulation of oestradiol in the culture medium by ovulatory (OF) and non-ovulatory (NF) follicles incubated with (▲) or without (●) testosterone (250 ng/ml). Bars bearing different letters (a, b, c, d, e) are significantly different ($P < 0{\cdot}05$). Because the data have been expressed as a geometric mean, individual values are shown.

ovulatory follicles in both groups secreted significantly more oestradiol into the culture medium than did non-ovulatory follicles ($P < 0{\cdot}05$). There was a significant correlation between in-vitro oestradiol secretion and the concentration of LH receptors in thecal ($r = 0{\cdot}86$; $P < 0{\cdot}01$; $n = 18$) and granulosa ($r = 0{\cdot}62$; $P < 0{\cdot}01$; $n = 17$) cells for all ovulatory follicles, but within groups this correlation was significant only for Group 1 ovulatory follicles ($r = 0{\cdot}72$; $P < 0{\cdot}05$; $n = 9$; $r = 0{\cdot}81$, $P < 0{\cdot}01$, $n = 9$ respectively). Ovulatory follicles in Group 1, but not Group 2, secreted significantly more testosterone than did non-ovulatory follicles ($P < 0{\cdot}05$). Furthermore, non-ovulatory follicles in Group 1 secreted significantly more testosterone than did non-ovulatory follicles in Group 2 ($P < 0{\cdot}05$) (Text-fig. 2c). The concentration of testosterone in the medium during the first 2 h of the incubation period was significantly correlated with LH receptor concentration in thecal cells only when data from all the follicles were considered ($r = 0{\cdot}51$; $P < 0{\cdot}01$; $n = 25$). The rate of testosterone secretion and LH receptor concentrations in granulosa cells were not significantly correlated. Addition of testosterone (250 ng/ml) to the culture medium during the final 2 h of the incubation period did not significantly alter the rate of oestradiol secretion in any of the follicles tested (Text-fig. 3) with the exception of Group 2 non-ovulatory follicles for which there was a significant increase.

Follicle size

The diameters of the ovulatory and non-ovulatory follicles did not change significantly in Groups 1 and 2 (Table 3). Ovulatory follicles were significantly larger than non-ovulatory follicles ($P < 0{\cdot}01$).

Table 3. Diameters of ovulatory and non-ovulatory follicles of sheep

	Follicular diam. (mm)	
	Ovulatory	Non-ovulatory
Group 1 ewes	*7·8 ± 0·7 (9)	3·7 ± 0·6 (24)
Group 2 ewes	*7·4 ± 0·5 (9)	2·9 ± 0·2 (26)

Values are mean ± s.e.m. for the no. of ewes indicated in parentheses.

* Significantly different from values for non-ovulatory follicles ($P < 0·01$).

Discussion

The results reported here extend the observation that the number of thecal and granulosa cell LH receptors in ovulatory follicles increase during the ascending limb of the preovulatory gonadotrophin surge (Webb & England, 1982), and demonstrate that after the peak of the preovulatory gonadotrophin surge there is a significant fall in the binding of ^{125}I-labelled hCG to the thecal and granulosa cells of ovulatory follicles (Text-fig. 2a) and in ovarian oestradiol production (Table 2) and peripheral oestradiol concentrations (Text-fig. 1). The fall in LH receptor capacity was significantly correlated with follicular fluid oestradiol concentrations (Text-fig. 2b) and in-vitro oestradiol production (Text-fig. 2c).

LH, but not FSH, stimulates oestradiol secretion following infusion (McCracken, Uno, Goding, Ichikawa & Baird, 1969), and an endogenous pulse of LH has been associated with an increase in circulating oestradiol concentrations (Baird, Swanston & Scaramuzzi, 1976; Baird, 1978; Baird, Swanston & McNeilly, 1981). Paradoxically, the administration of high doses of LH in the sheep inhibits oestradiol secretion both *in vivo* and *in vitro* (Rado, McCracken & Baird, 1970; Moor, 1974) and elicits a similar response in the rat (Hori, Ide & Miyake, 1969; Hori, Ide, Kato & Miyake, 1970). The mechanisms whereby LH enhances or inhibits follicular oestradiol secretion are not well understood; however, the presence of large concentrations of LH receptors in the membrana granulosa of the major oestradiol-secreting follicle (England *et al.*, 1981b; Webb & England, 1982) would suggest that their presence plays an important role in follicular oestrogen synthesis. Certainly non-ovulatory follicles which do not possess significant numbers of granulosa cell LH receptors (Text-fig. 2a) have a reduced capacity to produce oestradiol *in vitro* (Text-fig. 2c) and have lower follicular fluid oestradiol concentrations (Text-fig. 2b; Webb & England, 1982).

The importance of FSH in aromatization is not known because intact follicles do not show the significant increase in oestrogen production exhibited by cultured granulosa cell monolayers (Moor, 1977; Hay & Moor, 1978). In cows (Webb & Bellows, 1980) and rats (Uilenbroek & Richards, 1979) the fall in in-vitro oestradiol production during the preovulatory gonadotrophin surge was not correlated with a decrease in the number of FSH receptors. In certain physiological situations, therefore, FSH is not the major hormone controlling oestradiol production and the exact role that FSH plays in ovarian steroidogenesis in the ewe awaits further study.

Although non-ovulatory follicles have a reduced capacity to produce oestradiol they can secrete significant amounts of testosterone (Text-fig. 2c; England *et al.*, 1981b; Webb & England 1982). This may also involve LH as there was a significant correlation between thecal LH receptor and in-vitro testosterone production. Androgens seem to be produced by the thecal

compartment (Moor, 1977) and certainly in our study in-vivo (Table 2) and in-vitro (Text-fig. 2c) testosterone production and thecal cell LH receptors (Text-fig. 2a) in ovulatory and non-ovulatory follicles decreased after the peak of the preovulatory gonadotrophin surge.

Other factors in addition to the binding of LH undoubtedly play a role in the regulation of steroid production. Both FSH and LH have been shown to stimulate cAMP production (Kolena & Channing, 1972; Lindner *et al.*, 1974; Hunzicker-Dunn & Birnbaumer, 1976a, b; Weiss, Seamark, McIntosh & Moor, 1976) and there is support for the proposal that the stimulation of ovarian steroidogenesis by gonadotrophins is mediated by cAMP (Uilenbroek & Richards, 1979). Although a change in receptor capacity may play a major role in modulating follicular steroidogenesis, especially during the preovulatory gonadotrophin surge, decreasing receptor number has also been correlated with a loss of LH- and FSH-responsive adenylate cyclase (Hunzicker-Dunn & Birnbaumer, 1976a, b). The loss of responsive adenylate cyclase, or desensitization occurs faster than the loss of LH receptor after exogenous hormone treatment (Jonassen & Richards, 1980; Jaaskelainen, Hyvonen & Rajaniemi, 1980), and so one of the first steps in the decreased rate of oestrogen steroidogenesis during the preovulatory gonadotrophin surge may be a fall in cAMP production followed by a subsequent fall in receptor number.

The mechanisms that induce the fall in LH receptor numbers demonstrated in this study are not known. Rao, Richards, Midgley & Reichert (1977) have induced a fall or down regulation in the number of LH receptors on rat granulosa cells by giving injections of hCG. The decrease in binding was shown to be due to a decrease in the actual number of unoccupied receptors rather than to an increased occupancy of available receptors. If the same response occurs in sheep, then the fall in the number of LH receptors after the peak of preovulatory gonadotrophin surge is probably due to a decrease in the number of unoccupied receptors. This conclusion is supported by the finding that the number of unoccupied binding sites increases significantly before the peak of the gonadotrophin surge in spite of the large increase in peripheral LH concentrations during the surge (Webb & England, 1982).

Both testosterone (Moor, 1977; Hay & Moor, 1978; Scaramuzzi, Baird, Clarke, Martensz & Van Look, 1980; Webb & England, 1982) and androstenedione have been suggested as major precursors for oestradiol production (Rado *et al.*, 1970). In the present study, incubation of follicles with 250 ng testosterone/ml failed to alter in-vitro oestradiol production in all except Group 2 non-ovulatory follicles (Text-fig. 3), suggesting that the fall in oestradiol was not because of a lack of precursor (Baird *et al.*, 1981) and agrees with a similar study in the cow (Webb & Bellows, 1980). If the fall in oestradiol secretion is not attributable to a fall in available precursor then a possible cause may be an inactivation of the aromatase enzyme system. Indeed, evidence to suggest that testosterone was available to follicular cells is provided by the high follicular fluid testosterone concentrations measured in this study (Text-fig. 2c) when compared with previously reported values (England *et al.*, 1981a, b; Webb & England, 1982). Presumably the testosterone binds to a protein present in the follicular fluid (Cook, Hunter & Kelly, 1977; England *et al.*, 1981a). Follicles incubated with 250 ng testosterone/ml had follicular fluid testosterone concentrations 5–10 times higher than did follicles incubated with no testosterone (B. G. England & R. Webb, unpublished data), supporting the notion that the testosterone is passing through the thecal and granulosa cell layer into the follicular fluid.

In summary, the fall in oestradiol secretion rates that occur during the preovulatory gonadotrophin surge may be due to a decrease of aromatase activity associated with a fall in the concentration of LH receptors. The high testosterone and low oestradiol concentrations in non-ovulatory follicles are probably due to an inactive aromatase system, possibly being correlated with the lack of granulosa cell LH receptors.

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