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Changes in the Concentration of Gonadotrophic and Steroidal Hormones in the Antral Fluid of Ovarian Follicles throughout the Oestrous Cycle of the Sheep

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Abstract

The concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, progesterone, androstenedione and oestradiol were determined in the antral fluid of ovarian follicles >1 mm in diameter as well as in ovarian venous or peripheral venous plasma, or both, from at least four different animals on each day throughout the oestrous cycle of the sheep. The individual steroid hormones in antral fluid were examined in relation to the steroid-secretion rates in ovarian venous plasma, follicle size and the hormone levels in jugular venous plasma.

The range of levels of FSH, LH and prolactin in antral fluid was comparable to that in peripheral plasma. Irrespective of follicle size, the highest concentrations of FSH were present in follicles with high levels of oestrogen whereas the lowest were found in those follicles with low levels of oestrogen. In most follicles, the levels of LH were below 4 ng/ml but rose to high values at the time when a pre-ovulatory rise of LH was recorded in plasma. Prolactin was present in significantly higher concentrations in large follicles (≥ 5 mm diam.) compared with small follicles (>1 to <5 mm diam.) except during the follicular phase when there was a progressive decline in prolactin levels in large, but not small, follicles to low values before ovulation.

The steroid levels in antral fluid were up to 500 000 times higher than those in peripheral plasma. In most follicles progesterone concentration was normally below 100 ng/ml, but increased to about 700 ng/ml in large follicles (≥ 8 mm diam.) before ovulation and reached values of 2000 ng/ml in the newly formed corpus luteum. Androstenedione concentration in antral fluid varied between 2 and 70 ng/ml without any obvious pattern and the concentrations in large follicles (≥ 5 mm) were not significantly different from those in small follicles. Oestradiol concentration in antral fluid varied between 10 and 1000 ng/ml. There were higher levels in large follicles than in small follicles and peak concentrations occurred in large follicles just before the onset of oestrus and also in similar-sized follicles on the third day after oestrus.

It is suggested that the intrafollicular hormonal milieu may be important for the development of the antral follicle, maturation of the oocyte and the secretory status of the granulosa cells both before and after ovulation.

Introduction

The granulosa cells and oocyte of the antral follicle do not have direct access to a blood supply but are bathed in a fluid which contains most serum proteins, variable amounts of gonadotrophins and high concentrations of steroids (McNatty 1978). The endocrine micro-environment of the mammalian follicle has been correlated with the mitotic and biosynthetic activities of granulosa cells and the nuclear and cytoplasmic maturation of the oocyte (McNatty and Sawers 1975; Moor and Trounson 1977; Moor *et al.* 1978; McNatty *et al.* 1979*a*, 1979*b*). However, information on the hormonal milieu in antral fluid of sheep follicles of different sizes on different days of the ovarian cycle is limited (Edgar 1953; Brand 1970; McNatty and Gibb 1977; Moor *et al.* 1978).

In the sheep ovary, androstenedione and oestradiol are secreted mainly from large antral follicles (Hay and Moor 1975; Baird and Scaramuzzi 1976) whereas progesterone is secreted mainly from the corpus luteum (Baird *et al.* 1975). Therefore, the rate of androgen and oestrogen secretion into ovarian venous blood provides some indication of the level of follicular activity (Moore *et al.* 1969; Cox *et al.* 1971; Baird *et al.* 1976). However, the relationships between the steroids secreted by the follicles into the blood stream and those accumulated within the avascular follicular tissues are poorly understood.

In the present study, peripheral or ovarian venous blood, or both, and ovarian antral fluid were collected from ewes at varying stages of the ovarian cycle, and the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, androstenedione, oestradiol and progesterone were determined. In addition, the secretion rates of androstenedione and oestradiol by the individual ovaries were estimated before ovariectomy. These data were used to investigate the interrelationships among the concentrations of pituitary and steroid hormones in antral fluid, the level of follicular activity (as assessed by the steroid secretion rates into ovarian venous plasma), follicle size and the hormone levels in plasma.

Materials and Methods

Experimental Animals

One hundred and sixteen parous New Zealand Romney ewes (aged 2·5–4·5 years and bodyweight 38–66 kg) grazed on open pasture, were used in these studies. Most animals were recorded in oestrus on two previous occasions before undergoing surgery. Ewes were examined twice daily for oestrous activity detected by vasectomized rams fitted with marking harnesses. On the day before oestrus (i.e. 16 days after the previous day of oestrus; day −1) and on the expected day of oestrus (i.e. 17 days after the previous day of oestrus; day 0) the animals were examined for oestrous activity three to five times per day. The average cycle length preceding the one in which the animals underwent surgery was 17·1±0·1 days (mean ±s.e.m., 116 observations). The seventeenth day after previous day of oestrus was classified as day −0 if the animals had not displayed oestrous activity before surgery and as day +0 if the animals were in oestrus before surgery.

Jugular and ovarian venous blood samples, and ovarian tissues were collected from at least four different animals on each day of a complete 17-day ovarian cycle.

Recovery of Blood Samples, Ovarian Tissues and Antral Fluid

The ewes were anaesthetized by i.v. injection of 5% (w/v) sodium thiopentone (Intraval; May and Baker, New Zealand), and maintained in an anaesthetized state with either a halothane (Fluothane; ICI, New Zealand)–oxygen mixture (80 animals) or with additional sodium thiopentone when required (36 animals). A ventral abdominal incision was made and the uterus and ovaries exteriorized, taking care not to handle the ovaries. 25000 i.u. of heparin were administered to the animal via a jugular venous cannula and a 40-ml blood sample taken from the jugular vein. Before removal of the ovaries, the largest ovarian vein draining each ovary was cannulated using the largest vinyl tube (range 2·5–4·0 mm o.d.) capable of being accommodated by the vein. During the blood-collection period, any ovarian vein that was not cannulated was clamped so that the entire venous effluent could be collected over a known time period. The length of the cannula was reduced to a minimum, usually 150 mm, and up to 25 ml of blood was collected during a recorded interval into a heparinized volumetric flask. In some animals, jugular venous blood samples (10 ml) were taken once daily throughout one complete oestrous cycle before the one in which they underwent surgery. All blood samples were centrifuged (4000 *g* for 15 min at 6°C) immediately after collection and aliquots of the plasmas were recovered and frozen to −20°C for subsequent hormone determinations.

Immediately after ovariectomy, the ovaries were weighed and their gross morphology recorded. The excised tissues were then carefully dissected so that all antral follicles (>1 mm diam.), corpora lutea, corpora albicantiae and the stroma could be isolated and individually weighed. Subsequently,

the fluid from each follicle and the fluid in the central cavity of the corpus luteum were gently aspirated through a 26-gauge needle into a 1-ml syringe. Each fluid specimen was then transferred to a 0·5-ml plastic ampoule which was sealed and stored frozen at −20°C until assayed for hormones. No attempts were made to recover the oocytes or other individual ovarian cell types. Moreover, no attempt was made to assess whether the follicles were healthy or atretic.

Calculation of Ovarian Secretion Rates

The secretion rate of ovarian steroids was calculated from a knowledge of the time taken to collect a certain volume of blood (blood flow), the concentration of steroid in ovarian venous plasma and haematocrit. The haematocrits were determined in an MSE (Crawley, UK) Microhaemotocrit centrifuge (at 16000 rpm for 2 min) using heparinized capillary tubes (Terumo Corp., Tokyo, Japan). The results were not corrected for any steroid which may have been adsorbed onto the red-cell surfaces.

Hormone Assays

LH and FSH

The radioimmunoassays for LH and FSH in plasma and follicular fluid were based on those described previously (Scaramuzzi *et al.* 1970; Salamonsen *et al.* 1973). The pituitary LH preparation for both standard and the iodinated tracer was NIH-LH-S11 (biopotency 0·81 × NIH-LH-S1). The LH antibody, raised in a rabbit against NIH-LH-S11, was used at an initial dilution of 1 : 50000. The assays of LH in plasma were performed in duplicate whereas those in antral fluid were made on single samples. The volume of plasma used in the LH assay varied from 0·05 to 0·40 ml whereas the volume of antral fluid varied from 0·01 to 0·05 ml. The limit of detection for LH was 0·3 ng/ml in plasma and 2·5 ng/ml in antral fluid. In plasma, the within-assay c.v. was $<18\%$ over the range 0·3–250 ng/ml. All samples for LH analysis were measured in the one assay as were those for FSH and prolactin.

For the FSH assay, NIH-FSH-S6 (biopotency 1·24 × NIH-FSH-S1) was used as the standard, and a Papkoff preparation G4-150C (biopotency 54 × NIH-FSH-S1) for the iodinated tracer. Rabbit anti-human FSH (Butt, M94) (Lynch and Shirley 1974; McNeilly *et al.* 1976) was used at an initial dilution of 1 : 4000. Because insufficient antral fluid was available for individual determinations of FSH, the samples were pooled after first determining the levels of oestradiol and androstenedione. Those samples which contained an oestradiol : androstenedione ratio >1 were pooled, as were those with a ratio <1. In addition, fluids from individual corpora lutea were pooled so that volumes of at least 0·1 ml were obtained. With a 0·1-ml sample of antral fluid or plasma the limit of detection for FSH was 10 ng/ml. The within-assay c.v. was $<15\%$ over the range 10–75 ng/ml.

Prolactin

The radioimmunoassay for prolactin in plasma and antral fluid was based on that described by McNeilly (1970) and Lamming *et al.* (1974). Ovine prolactin, NIH-P-S11 (biopotency 1·32 × NIH-P-S1), was used as the reference standard and ovine prolactin, LER-860-2 (30 i.u./mg), for iodination. The antiserum, which was raised against ovine prolactin (NIH-P-S6), was used in the assay at a dilution of 1 : 50000. The volume of antral fluid in the assay was usually 10 μl whereas the volume of plasma varied from 10 to 50 μl. The assays of prolactin in plasma were made in duplicate whereas those in antral fluid were made on single samples. The limit of detection for prolactin in antral fluid and plasma was 10 ng/ml. For plasma, the within-assay c.v. was $<11\%$ over the range 10–150 ng/ml.

Progesterone

Progesterone was measured in peripheral plasma using a radioimmunoassay procedure similar to that described by Thorneycroft and Stone (1972) and in antral fluid by the method of Neal *et al.* (1975). Antral fluid (1–5 μl) was diluted 100- to 1000-fold in phosphate-buffered saline (0·1 M, pH 7·0) and 0·1-ml samples were assayed without extraction. The precision and accuracy of the assay were similar to those described by Neal *et al.* (1975). The progesterone antiserum (WA-26) was raised in an ovariectomized ewe against progesterone-11α–hemisuccinate conjugated to bovine serum albumin and used at initial dilution of 1 : 10000. Major cross-reactions of the steroids in

the assay were 11α-hydroxyprogesterone 120%, 11β-hydroxyprogesterone 25%, 20α-hydroxyprogesterone 3·5% and androstenedione 0·45%. Plasma samples were assayed in duplicate and, on most occasions, samples of antral fluid were assayed singly due to the limited amount of fluid available. The minimum detectable level of progesterone in plasma was 0·3 ng/ml and in antral fluid 10 ng/ml.

Androstenedione

Androstenedione in plasma was measured using a radioimmunoassay technique reported elsewhere (McNatty *et al.* 1976). However, the ether-extracted steroid fraction was subjected to a Lipidex column separation rather than the alumina column separation technique described by McNatty *et al.* (1976). The ether fraction, which also contained the extracted recovery counts of [1,2-^{3}H]-androstenedione, was evaporated to dryness and dissolved in 0·2 ml of a hexane–chloroform mixture (80/20, v/v). This fraction was then added to a 2·5-ml glass syringe barrel which contained 0·5 g of Lipidex-5000 supported on a disc of glass-fibre filter paper. The androstenedione was collected in a 1·0-ml eluate of hexane–chloroform (80/20, v/v) after the first 1·5 ml of eluate was discarded. The antiserum for the assay was raised in an ovariectomized ewe against the conjugate 11α-hydroxy-4-androstene-3,17-dione–hemisuccinate coupled to bovine serum albumin. The antiserum (WA-965) was used in the assay at an initial dilution of 1 : 3000. Cross-reactions of other steroids with this antiserum were similar to those described by McNatty *et al.* (1976). Androstenedione in antral fluid (1–10 μl) was measured in unextracted, diluted (50- to 1000-fold) aliquots since the results obtained were comparable to those subjected to Lipidex column chromatography. The assays in ovarian venous plasma were carried out in duplicate and those for antral fluid were most commonly made on single samples. The limit of detection for androstenedione in plasma was 15 pg/ml and in antral fluid 2 ng/ml.

Oestradiol

Oestradiol in plasma was assayed by a method similar to that described by Baird *et al.* (1976) except that the ether-extracted steroid fraction was subjected to a Sephadex LH-20 column procedure. The ether fraction, which also contained the extracted recovery counts of [2,4,6,7-^{3}H]oestradiol, was evaporated to dryness and dissolved in 0·2 ml of a benzene–methanol mixture (85/15, v/v). This mixture was then added to the top of a Sephadex LH-20 column (0·4 g LH-20 in 2·5-ml glass syringe barrel and supported by a glass-fibre paper), and the resulting eluate discarded. The oestradiol fraction was eventually collected in a 2·0-ml benzene–methanol fraction (85/15, v/v) after an earlier 1·4 ml of eluate had also been discarded. Oestradiol in antral fluid (1–5 μl) was measured in unextracted, diluted (100- to 1000-fold) aliquots. The antiserum (WA-27) used in the assay was raised in an ovariectomized ewe against oestradiol-6-(*O*-carboxymethyl)oxime bovine serum albumin conjugate used at an initial dilution of 1 : 16000. The major cross-reactions of other steroids in this assay were oestrone, 7·3%; oestriol, 1·4%; oestradiol-17α, 1·4%; androstenedione, 0·015%.

The assays of plasma samples were carried out in duplicate while samples of antral fluid were normally assayed singly. The usual minimum detectable level of oestradiol in plasma and antral fluid was 5 and 500 pg/ml respectively.

Statistics

Unless stated otherwise, the data were subjected to either paired or unpaired Student's *t*-test analyses.

Results

When concentrations of hormones in peripheral plasma or follicular fluid of sheep anaesthetized with halothane were compared with those treated with sodium thiopentone no significant differences could be noted. The results from all ewes were therefore pooled. Of the 116 animals studied, 14 had two corpora lutea and the remainder one corpus luteum.

Hormones in Peripheral Plasma

Mean values (+s.e.m.) obtained for the concentrations of LH, FSH, prolactin, oestradiol and progesterone in peripheral plasma just before ovariectomy with respect to the day of the ovarian cycle are shown in Fig. 1. The concentrations of progesterone in peripheral plasma of eight ewes during the ovarian cycle preceding surgery are also shown in Fig. 1.

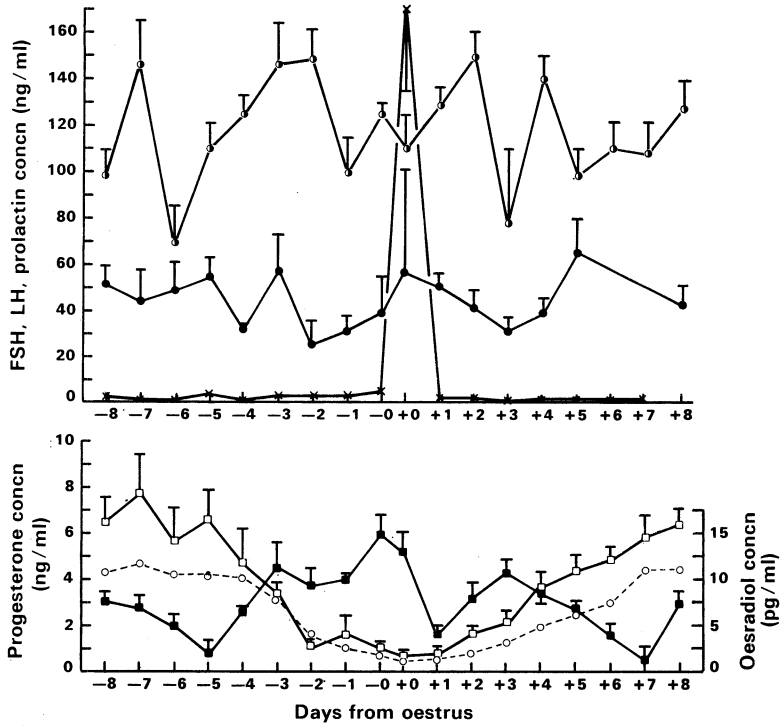

Fig. 1. Daily concentrations (ng/ml) of LH (×), FSH (●), prolactin (◑), oestradiol (■) and progesterone (□ or ○) in peripheral plasma of ewes undergoing surgery, with respect to the day of oestrous cycle. Values are expressed as mean +s.e.m. For all hormones each point represents the mean of at least three observations. The presurgical values (○---○) for progesterone represent those levels in the cycle before the one (□) in which the animals underwent surgery ($n = 8$). The seventeenth day after the previous oestrus was classified day −0 if ewes had not exhibited oestrus prior to surgery; if ewes displayed oestrus before surgery, the day was classified +0.

Secretion Rates of Androstenedione and Oestradiol

The rate of blood flow varied markedly between the left and right ovaries and also between animals (i.e. from 1 to 20 ml/min). Most of the blood flows were recorded between 1 and 10 ml/min. Only five of the measured flow rates exceeded 10 ml/min: two of these were for ewes on day 3 of the cycle, one was for a ewe on day −0 and the other was for a ewe on day −7. The mean (±s.e.m.) haematocrit of the ovarian venous blood samples was 33·9±0·6%. The range of concentrations for oestradiol in ovarian venous plasma was 30–1290 pg/ml, and for androstenedione 40–5460 pg/ml.

Since the individual blood-flow measurements recorded for animals anaesthetized with halothane were within the same range of values recorded for those on sodium thiopentone (see Mattner *et al.* 1976), the data were grouped according to follicle size and day of cycle.

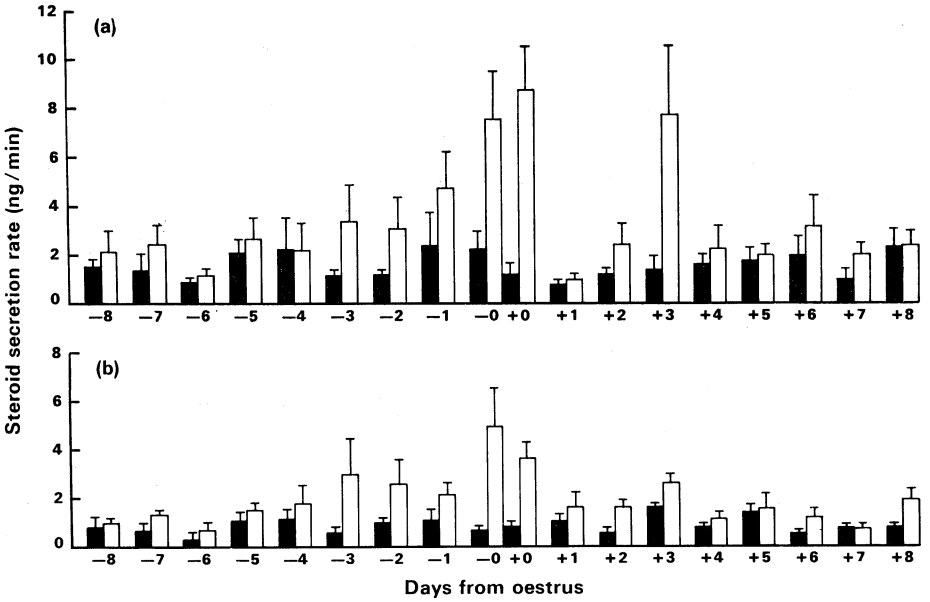

Fig. 2. Secretion (ng/min) of androstenedione (*a*) and oestradiol (*b*) from ovaries containing large (open histograms, ⩾5 mm diam.) or small (solid histograms, >1 to <5 mm diam.) antral follicles with respect to the day of oestrous cycle. Values represent means +s.e.m. Each histogram represents the mean of 4–16 observations. The seventeenth day after the previous oestrus was classified day −0 if ewes had not exhibited oestrus prior to surgery; if ewes displayed oestrus before surgery, the day was classified +0.

The secretion rates of androstenedione and oestradiol from ovaries containing large (⩾5 mm diam.) or small (<5 mm diam.) follicles throughout the ovarian cycle are shown in Fig. 2. Throughout the cycle the respective secretions of androstenedione and oestradiol from the ovaries containing a large follicle(s) (⩾5 mm diam.) were significantly greater than those from ovaries containing small follicles (<5 mm diam.) ($P < 0{\cdot}005$ respectively). Also throughout the cycle, the secretion of androstenedione was significantly greater than that of oestradiol (from ovaries with large follicles, $P < 0{\cdot}001$; from ovaries with small follicles, $P < 0{\cdot}001$). Peak secretion rates of both androstenedione and oestradiol were recorded on the day of oestrus (day 0). Also increased secretions of androstenedione and to a lesser extent of oestradiol were recorded on day 3; however, these were not statistically significant. From ovaries containing a large follicle (⩾5 mm diam.) there was a significant linear correlation between the secretion of oestradiol and androstenedione ($r = 0{\cdot}7956$, $P < 0{\cdot}001$, linear regression analysis) throughout the oestrous cycle. By contrast, from ovaries with only small follicles (<5 mm diam.), there was no significant correlation between the secretion of oestradiol and androstenedione ($r = 0{\cdot}3645$, $P > 0{\cdot}05$; linear regression analysis).

Intra-ovarian Studies

There was a significant correlation between the weight of follicular tissue (>1 mm diam.) and the weight of the ovaries throughout the oestrous cycle (for ovaries with corpus luteum: $r = 0{\cdot}82$, $P < 0{\cdot}01$; for ovaries without corpus luteum: $r = 0{\cdot}52$, $P < 0{\cdot}01$; linear regression analysis). The mean weight of follicles >1 mm in the ovary with a corpus luteum was significantly greater than on the contralateral ovary (0·62 *v.* 0·49 g, $P < 0{\cdot}02$). In the ovary without luteal tissue, the follicular

Table 1. Concentration of gonadotrophins and steroids in individual follicular luteal and stromal fluids in a sheep ovary on day +4 of the oestrous cycle

Stromal fluid was obtained from minced stromal tissues devoid of antral follicles (>0·5 mm diam.) and luteal tissue. Luteal fluid was obtained from the central cavity of the corpus luteum. Blood sample was taken just prior to ovariectomy on day +4 of the cycle. — No measurement made

Ovarian tissue	Hormone concentration (ng/ml)				
	LH	Prolactin	Progesterone	Androstenedione	Oestradiol
Follicle					
7 mm diam.	2·2	50	30	3	330
6 mm diam.	1·0	75	70	11	222
5 mm diam.	1·0	140	36	11	62
3 mm diam.	—	—	14	13	22
Stroma	—	—	230	23	3
Corpus luteum (8 mm diam.)	—	140	1820	10	11
Peripheral blood	3·5	96	1·5	0·035	0·016

mass constituted about 54% of the total ovarian weight, whereas in the ovary with luteal tissue the follicular mass was about 40% of the total ovarian weight. At least four (range 4–15) antral follicles >1 mm in diameter were present in each ovary on each day of the ovarian cycle. The overall mean number of follicles (>1 but <5 mm diam.) in the ovary with a corpus luteum was $9{\cdot}1 \pm 0{\cdot}3$, whereas in the ovary without a corpus luteum it was $7{\cdot}9 \pm 0{\cdot}3$ ($P < 0{\cdot}05$). On each day of the cycle there was at least one, but never more than three, antral follicle ⩾5 mm in diameter present on one or both ovaries.

Table 2. Concentrations of FSH in follicular fluid, luteal fluid and peripheral blood

FSH concentration is given as mean value or range

Fluid	FSH concn (ng/ml)	Fluid	FSH concn (ng/ml)
Follicular fluid		Luteal tissue fluid	63·0
(1)[A]	71·5	Peripheral plasma	30–66,
(2)[B]	26·3		mean = 46

[A] Fluid from follicles with an oestradiol : androstenedione ratio >1. FSH determined from a 'fluid-pool' from 52 follicles.

[B] Fluid from follicles with an oestradiol : androstenedione ratio <1. FSH determined from a 'fluid-pool' from 16 follicles.

The concentrations of pituitary and steroid hormones in the follicular, luteal and stromal fluid compartments of individual ovaries were found to be different from one another. For example, data obtained for one ovary are shown in Table 1.

Hormones in Follicular Fluid

FSH

The results are summarized in Table 2. The level of FSH in antral fluid was correlated with the relative amounts of oestradiol and androstenedione present. The level of FSH in luteal fluid was comparable to that in peripheral blood.

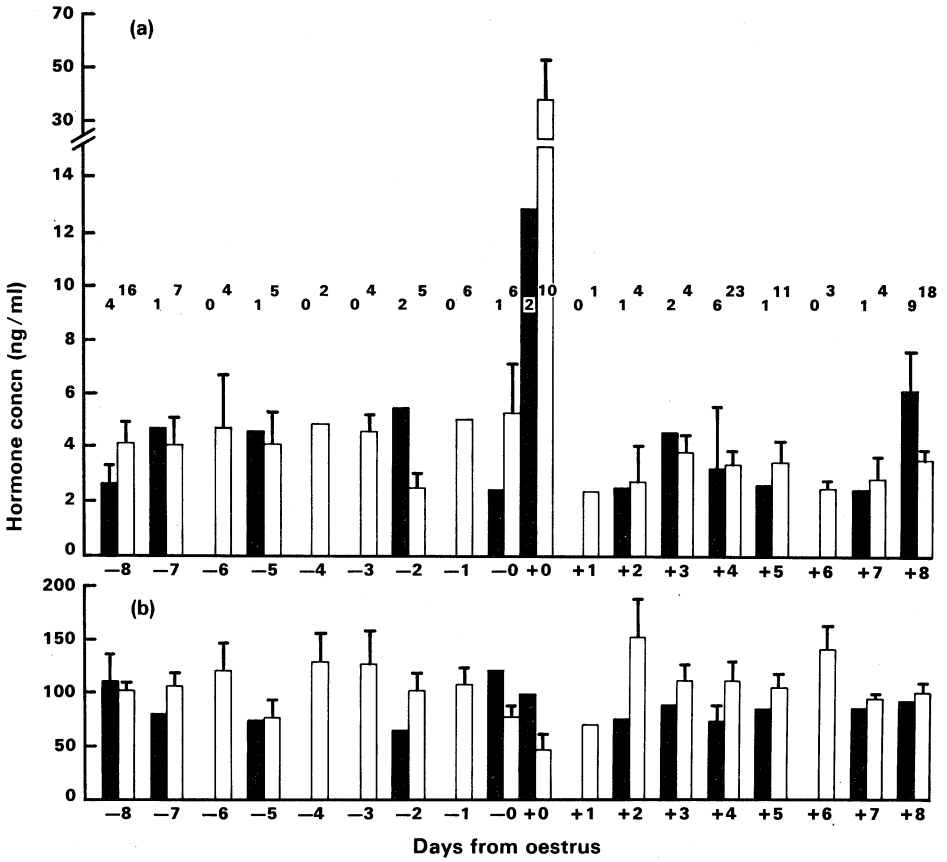

Fig. 3. Concentration (ng/ml) of luteinizing hormone (LH) (*a*) and prolactin (*b*) in the same samples of antral fluid of large (open histograms, ⩾5 mm diam.) and small (solid histograms, >1 to <5 mm diam.) follicles with respect to the day of oestrous cycle. Values represent means +s.e.m. Number of follicles tested for both LH and prolactin are indicated above the histograms in (*a*). The seventeenth day after the previous oestrus was classified day −0 if ewes had not exhibited oestrus prior to surgery; if ewes displayed oestrus before surgery, the day was classified +0.

LH and prolactin

The mean concentrations of LH and prolactin in antral fluid with respect to follicle size and the stage of the ovarian cycle are shown in Figs 3*a* and 3*b*. LH was detectable in most (~86%) follicles examined (>2·5 ng/ml). Overall, the levels of LH in small follicles (<5 mm diam.) were not significantly lower or higher than those in the large follicles (⩾5 mm diam.) ($P > 0{\cdot}05$). The highest levels of LH were recorded in large follicles (⩾5 mm diam.) recovered during behavioural oestrus (day +0); the mean level in such follicles at this time was significantly higher than

that in large follicles on day −0 (i.e. before behavioural oestrus) ($P < 0{\cdot}05$) and moreover, it was significantly higher than that in other large follicles throughout the remainder of the cycle ($P < 0{\cdot}05$). On day −0, the mean level of LH in large follicles was ~45% of that recorded in plasma. However, for all other days of the cycle (i.e. days −8 to −1, and days +1 to +8) the mean level of LH in large follicles was significantly higher than in peripheral plasma (i.e. antral fluid, 3·7 ng/ml; plasma, 2·0 ng/ml; $P < 0{\cdot}001$). Likewise for small follicles, the overall mean level in antral fluid was significantly higher than in plasma (3·76 *v.* 1·67; $P < 0{\cdot}01$).

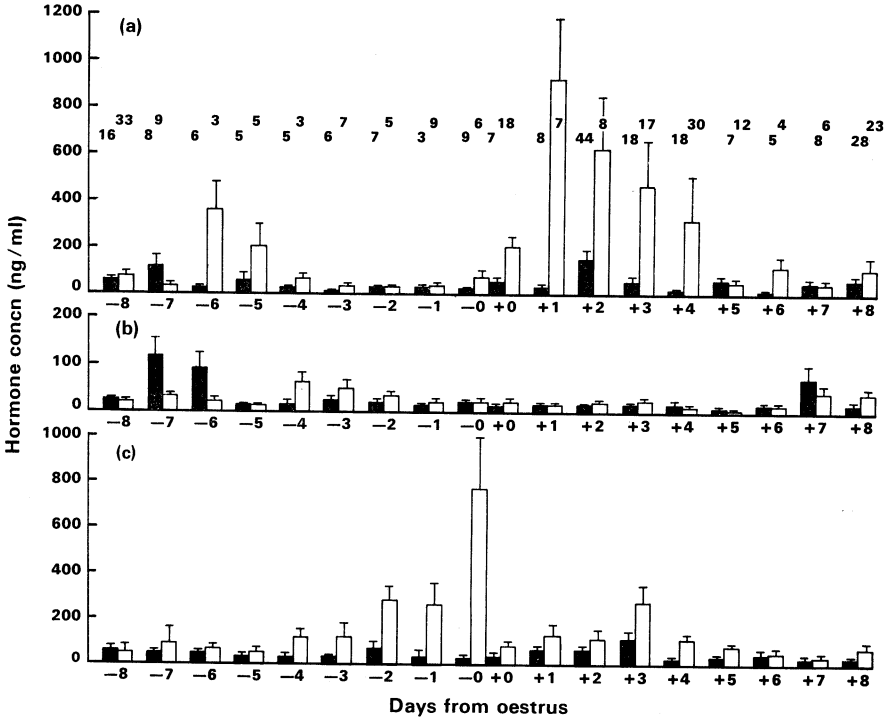

Fig. 4. Concentration (ng/ml) of progesterone (*a*), androstenedione (*b*) and oestradiol (*c*) in the same samples of antral fluid of large (open histograms, ⩾5 mm diam.) and small (solid histograms, >1 to <5 mm diam.) follicles with respect to the day of the oestrous cycle. Values represent means +s.e.m. Number of follicles tested for all steroids are listed above the histograms in (*a*). The seventeenth day after the previous oestrus was classified day −0 if ewes had not exhibited oestrus prior to surgery; if ewes displayed oestrus before surgery. the day was classified +0.

Prolactin was detected in all the follicles examined. When the data on day 0 are excluded, the level of prolactin in small follicles was significantly lower overall than those in large follicles ($P < 0{\cdot}02$). In large follicles, the lowest mean concentration of prolactin was recorded around the time of behavioural oestrus. During the pre-ovulatory period, days 0 to +1, the mean level of prolactin in large follicles was significantly lower than the overall mean in the large follicles on the other days of the cycle ($P < 0{\cdot}01$). Overall, the respective mean levels of prolactin in small and large follicles were significantly lower than those in peripheral plasma ($P < 0{\cdot}05$, $P < 0{\cdot}05$ respectively).

Progesterone, androstenedione and oestradiol

The concentrations of these steroids in the same samples of antral fluid with respect to size of follicle and day of the ovarian cycle are shown in Figs 4*a*, 4*b* and 4*c*. The concentrations of progesterone, androstenedione and oestradiol in antral fluid were respectively between 2 and 1000, 250 and 500 (plasma values for androstenedione ranged from 0·020 and 0·300 ng/ml, data not shown) and 750 and 500 000 times higher than their corresponding range of values in peripheral plasma (compare Figs 1 and 4). The concentrations of progesterone and oestradiol in large follicles ($\geqslant$5 mm diam.) were significantly greater than their respective concentrations in small follicles (<5 mm diam.) ($P < 0{\cdot}01$ for both steroids). For androstenedione, the concentrations in large follicles were not significantly different overall from those in small follicles ($P > 0{\cdot}1$). Throughout the oestrous cycle, the concentrations of progesterone in antral fluid exceeded those of androstenedione in large follicles ($P < 0{\cdot}01$) but not in small follicles ($P > 0{\cdot}05$). The concentration of progesterone in large follicles was significantly higher than in small follicles on day +0 and also higher than in all follicles on day −1 ($P < 0{\cdot}05$). On day +1, when ovulation occurred, the levels of progesterone in antral fluid of large follicles were significantly higher than in follicles of comparable size on day +0 ($P < 0{\cdot}01$). The high levels of progesterone in large follicles on days +2, +3 and +4 were present when either the same or contralateral ovary contained a corpus luteum.

The concentrations of androstenedione in the antral fluid of large or small follicles did not correlate with those in the respective ovarian venous blood samples ($P > 0{\cdot}1$, $P > 0{\cdot}1$, linear regression analysis). Throughout the cycle, the concentrations of oestradiol in antral fluid exceeded those of androstenedione (large follicles, $P < 0{\cdot}01$; small follicles $P < 0{\cdot}01$). The changes in oestradiol concentrations in large follicles throughout the cycle correlated significantly with those in ovarian venous blood draining ovaries with large follicles ($P < 0{\cdot}001$, $r = 0{\cdot}7182$, linear regression analysis) and with those in peripheral blood ($P < 0{\cdot}001$, $r = 0{\cdot}6977$, linear regression analysis). By contrast, the changes in oestradiol concentrations in small follicles did not correlate with those in ovarian venous blood draining ovaries with small follicles ($P > 0{\cdot}05$, $r = 0{\cdot}3245$) or with those in peripheral blood ($P > 0{\cdot}1$, $r = 0{\cdot}1043$). On day −0, there were peak levels of oestradiol in the antral fluid of large follicles; these levels were significantly higher than those on days −1 or +0 ($P < 0{\cdot}05$ for day −0 *v.* −1 and $P < 0{\cdot}01$ for day −0 *v.* +0). There were also elevated levels in large follicles on day +3: these levels were significantly higher than those on day +4 ($P < 0{\cdot}01$) but not than those on day +1 or +2 ($P > 0{\cdot}05$, $P > 0{\cdot}05$ respectively).

Discussion

The levels of LH, FSH and oestradiol in peripheral plasma (Fig. 1) were similar to those described for conscious unstressed animals during the oestrous cycle (Salamonsen *et al.* 1973; Lamming *et al.* 1974; Yuthasastrakosol *et al.* 1975; Pant *et al.* 1977). Moreover, the ovarian secretions of androstenedione and oestradiol were not seriously affected by the surgical procedures since the secretion rates of these steroids were comparable to those reported in conscious animals (Baird and Scaramuzzi 1976; Baird *et al.* 1976; Baird 1978). By these criteria it is reasonable to assume that the intrafollicular levels of LH, FSH, androstenedione and oestradiol

were not affected by the sampling procedures. However, it is likely that the blood levels of progesterone and prolactin were affected by surgery since progesterone was present at lower concentrations in the same animals during the previous cycle (Fig. 1) and the levels of prolactin were consistently higher than those reported for unstressed Romney ewes (Munro *et al.* 1980).

In most instances, the levels of the gonadotrophins in antral follicles were comparable to those in plasma (Table 1, Table 2, Figs 3*a* and 3*b*). Presumably the high levels of prolactin in antral fluid were due to the elevated levels of this hormone in circulating blood (Fig. 1). Irrespective of the hormone levels in plasma, these studies demonstrate that no two follicles within the sheep ovary appear to contain the same milieu of gonadotrophins and steroids (Table 1).

The level of FSH within follicles correlated with the functional status of the follicles as judged by the antral-fluid levels of androgen and oestrogen. These studies report a higher level of FSH in 'oestrogenic' follicles (i.e. those with more oestradiol than androstenedione) than in 'androgenic' follicles (i.e., those with more androstenedione than oestradiol). Carson *et al.* (1979) have reported higher levels of FSH binding to granulosa cells in morphologically healthy follicles than in atretic follicles. Presumably, it is the FSH–oestrogen-'primed' follicle which is capable of further maturation whereas the FSH–oestrogen-depleted follicle is the one most likely to undergo or complete the atretic process (Carson *et al.* 1979).

These studies support the notion that the large antral follicle ($\geqslant 5$ mm diam.) is an important, if not the major, source of the androstenedione and oestradiol entering ovarian venous blood throughout the cycle (Baird *et al.* 1975; Baird and Scaramuzzi 1976). Although ovaries containing only small follicles actively secrete androstenedione and oestradiol into ovarian venous blood, it cannot be concluded that the follicular structures are the major ovarian sources of these steroids since no obvious correlation existed between the number (data not shown) or size of the small follicles and the concentration of androstenedione and oestradiol in ovarian venous plasma. Although significantly more androstenedione than oestradiol was secreted from the ovaries, there were significantly lower levels of androstenedione than oestradiol within the follicles irrespective of whether they were small (<5 mm diam.) or large ($\geqslant 5$ mm diam.) structures. Moreover the levels of androstenedione in the antral fluid of follicles did not correlate with those in ovarian venous blood. These findings are consistent with the concept that most of the androstenedione accumulating within the follicle is metabolized to testosterone by an oxido–reductase system (Moor *et al.* 1978), to oestrogen by the granulosa cells 'primed' with FSH, or both (Table 2).

The levels of oestradiol in large antral follicles correlated significantly with both the secretion rates of this hormone into ovarian venous blood and the levels of this hormone in peripheral blood. These data provide direct *in vivo* evidence that the level of oestradiol in blood is an indicator of steroidogenic activity in large antral follicles. Land (1973) and Findlay and Cumming (1977) have provided evidence to suggest that the emergence of a dominant follicle for ovulation may occur on day 14 (i.e. day -3). Presumably the dominant 'oestrogenic' follicle present after day -3 is the one which would normally go on to ovulate. On days -2 and -1, 14 large follicles ($\geqslant 5$ mm diam.) from 11 ewes were examined and eight of the follicles had oestradiol concentrations >200 ng/ml whereas only one of the ten small follicles (<5 mm diam.) examined from the above ewes had a level of oestradiol >200 ng/ml. On day -0, all large follicles (i.e. at least one large follicle per ewe) had concentrations

>200 ng/ml whereas all the small ones had concentrations <50 ng/ml (see Fig. 4*c*). These data suggest that on day −2, the presumptive preovulatory follicle is already quite large (~5 mm) and is capable of synthesizing large amounts of oestradiol.

Within the sheep follicle before ovulation, a significant rise in oestradiol concentration was recorded on day −0 and this was followed by a rapid decline to low levels later on the same day (i.e. day +0) when LH was elevated in both plasma and antral fluid (Fig. 3). These data suggest that the granulosa cells and oocyte in the sheep follicle are exposed to peak levels (>500 ng/ml) of oestradiol for less than 24 h. This is in contrast with some other species, such as man, in which the levels of oestradiol in preovulatory follicles remain elevated (>1 μg/ml) for several days (McNatty 1978). In sheep the significance of peak levels of oestradiol in antral fluid in relation to follicle maturation remains to be evaluated. Of interest is the presence of a second major peak of androstenedione secretion from the ovary containing a large follicle (≥5 mm diam.) on day +3 and high levels of oestradiol in the large follicle at this time (Figs 2 and 4). However, the significance of these ovarian events at this time of the cycle are unknown.

From the onset of luteal regression (day −4 to −3), the levels of progesterone in follicular fluid remained low until the onset of behavioural oestrus (day +0). On day +0, the progesterone levels rose significantly in the large, but not the small, follicles, concomitant with the appearance of high levels of LH and a reduction in those of prolactin (Figs 1, 2 and 4). A reduction in the follicular levels of prolactin concomitant with the pre-ovulatory rise in follicular progesterone has also been reported for human follicles but its significance is not understood (McNatty *et al.* 1975). It seems likely that the levels of progesterone continue to rise during the pre-ovulatory period since the fluid levels in the newly formed corpus luteum on day 2 (~2 μg/ml) were comparable to those found in the fully formed gland at mid-cycle (McNatty, unpublished data). Of interest is the finding that the large follicles present during the early luteal phase (days +2 and +3) also contained high levels of progesterone in antral fluid. It is possible that these follicles had not completed their maturation at the time of the LH surge but subsequently underwent luteinization in response to the LH which had accumulated within the follicle several days earlier. Clear evidence of morphological luteinization (data not shown) was observed in those follicles with the high levels of progesterone in antral fluid. Presumably, such follicles would eventually degenerate, or were already in the process of degeneration.

An important limitation of this study is that no assessment of follicular well-being was made in conjunction with the hormone measurements in antral fluid. Nevertheless it is reasonable to assume that the follicles with high levels of oestradiol (>250 ng/ml) were healthy in terms of their granulosa-cell number and oocyte status and that such follicles would probably have a potential for further maturation *in vivo* (McNatty *et al.* 1979*a*; McNatty, unpublished data). If this assumption is valid then it seems reasonable to suggest that a sequence of hormonal changes occurs within the microenvironment of the developing antral follicle in sheep similar to that demonstrated in human ovaries (McNatty *et al.* 1979*b*). The presence of high levels of FSH within the follicle may be important for its further development. The FSH-enriched follicles are those which develop the greatest capacity to generate oestradiol and such follicles are the major source of the oestradiol in ovarian venous blood. Presumably, it is the increased secretion of oestradiol from the dominant 'oestrogenic' follicle (and the subsequent pre-ovulatory discharge of LH) which leads to the accumulation

of LH and progesterone and the concomitant reduction in oestradiol and prolactin in the same follicle. This sequence of endocrine events within the avascular follicular tissues may be of considerable importance for the successful maturation of the dominant antral follicle during its final growth stages, and for its secretory activity after ovulation.

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