

Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary

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Summary. The patterns of ovarian follicular development and the steroidogenic properties of individual follicles (≥ 2 mm diam.) were assessed in Angus cows from Day – 5 until Day + 1 of the oestrous cycle (oestrus = Day 0). Individual follicles were judged to be healthy or atretic using a new classification system incorporating assessments of thecal vascularity and colour, the number of granulosa cells, the presence or absence of debris in follicular fluid and the status of the oocyte.

The results suggest that the theca interna of small antral follicles (< 5 mm diam.) responds to LH and synthesizes androstenedione before the granulosa cells develop an appreciable ability to metabolize androgen to oestrogen. Regardless of follicle size, the output of thecal androstenedione per unit mass of tissue remained unchanged in healthy but not in atretic follicles. On a per cell basis, aromatase activity increased in granulosa cells from healthy but not from atretic follicles with increasing follicle size. Peak levels of aromatizing activity were consistently observed in dominant oestrogen-enriched follicles on Day 0 although similar activity was also observed in some healthy follicles (≥ 8 mm diam.) on other days of the cycle. Early atresia in bovine follicles was characterized by an absence or lowering of aromatase activity in granulosa cells which always preceded any reduction in the thecal steroidogenic response to LH.

It was estimated that between 20 and 60 antral follicles (≥ 2 mm diam.) per cow may respond to LH by synthesizing androgen whereas only 1–3 follicles (> 5 mm diam.) have granulosa cells capable of metabolizing androstenedione or testosterone to oestradiol.

Introduction

It is generally accepted that bovine antral follicles grow and regress continuously throughout the oestrous cycle (Rajakoski, 1960; Donaldson & Hansel, 1965; Erickson, 1966; Priedkalns, Weber & Zemjanis, 1968). It has been estimated that a bovine follicle takes about 22 days to grow from 0.4 mm (i.e. early antral) to 10 mm (i.e. preovulatory size) in diameter, and more than 4 days to enlarge from 5 mm to preovulatory size (Scaramuzzi, Turnbull & Nancarrow, 1980; Staigmilller & England, 1982). From Day 4 of the oestrous cycle until ovulation there is usually one large follicle (≥ 8 mm diam.) present every day (Rajakoski, 1960; Choudary, Gier & Marion, 1968; Ireland, Coulson & Murphree, 1979; Matton, Adalakoun, Couture & Dufour, 1981; Ireland & Roche, 1983). From histological criteria it is thought that the number of non-atretic follicles > 5.0 mm in diameter is low (≤ 3 per cow) due to a high incidence of atresia in follicles enlarging from 2 to 5 mm in diameter (Rajakoski, 1960; Marion, Gier & Choudary, 1968; Scaramuzzi *et al.*, 1980).

Androstenedione, testosterone and oestradiol are major secretory products of bovine follicles and their concentrations in peripheral plasma fluctuate markedly, and often asynchronously, both within days and between each day of the oestrous cycle (Dobson & Dean, 1974; Lemon & Saumande, 1974; Peterson, Fairclough, Payne & Smith, 1975; Peterson, Fairclough & Smith, 1978; Wise, Caton, Thatcher, Lehrer & Fields, 1982). However, it has been difficult to extrapolate from hormone concentrations in peripheral plasma, the degree of follicular activity within the ovary. This is in part due to the fact that the relationship between the health of a follicle and its potential to synthesize steroids is poorly understood (Merz, Hauser & England, 1981; Kruij & Dieleman, 1982). Nevertheless, it is well established that large healthy (≥ 8 mm diam.) and/or preovulatory bovine follicles may synthesize large quantities of oestrogen (Short, 1962; Lunaas, 1964; England, Karavolas, Hauser & Casida, 1973; Merz *et al.*, 1981; Staigmiller, England, Webb, Short & Bellows, 1982; Kruij & Dieleman, 1982). For optimal oestradiol biosynthesis it is thought that a functional interaction between theca interna and granulosa cells is required with the two key variables being the amount of LH-induced androstenedione or testosterone synthesized by the theca interna cells and the level of aromatase activity in granulosa cells (Hillier, 1981; McNatty *et al.*, 1984a).

The objectives of this study were to determine (1) the degree of antral follicle development (≥ 2 mm diam.) from Day -5 until Day $+1$ of the oestrous cycle (oestrus = Day 0) and (2) the LH responsiveness of theca interna cells and amount of extant (or residual) aromatase activity in granulosa cells from healthy and atretic follicles.

Materials and Methods

Animals, blood sampling and recovery of ovaries. Thirty-three Angus cows aged 2–8 years (mean 4.7 years) were studied. Six animals were parous and 27 nulliparous. These animals were grazed on open pasture with a vasectomized bull and examined twice daily for oestrous activity. The mean oestrous cycle length (20.6 ± 0.2 days) in all 33 cows was determined from 7 ± 0.5 (s.e.m., $n = 33$) consecutive oestrous cycles. For each cow the mean \pm s.e.m. coefficient of variability with respect to cycle length was $8.4 \pm 0.9\%$ ($n = 33$). After monitoring oestrous cycle activity, 10 of the cows were slaughtered on a known day of the oestrous cycle at a local slaughterhouse, and the ovaries recovered within 30 min of slaughter. The remaining 23 animals underwent ovariectomy on a known day of the oestrous cycle at the Wallaceville Animal Research Centre, after blood had first been obtained from the jugular and ovarian veins (~ 20 ml/per vein/cow) of most animals. Ovariectomy and blood sampling was performed while the animals were under thiopentone sodium anaesthesia (Intraval; May and Baker, New Zealand). The ovaries were obtained within 1 h of induction of anaesthesia. All blood samples were collected into heparinized centrifuge tubes and centrifuged at 4000 g for 30 min at 6°C within 1 h of collection. Aliquots of the plasma were frozen to -20°C for subsequent hormone determinations.

From all cows, the ovaries were collected into chilled Medium A (Medium 199 containing Earle's salts, L-glutamine (0.1 g/l), HEPES buffer (20 mmol/l) (Gibco, Santa Clara, CA, U.S.A.) and 0.1% BSA (w/v, fraction V, Sigma Chemical Co., St Louis, MO, U.S.A.).

Ovarian studies. Excised ovaries were weighed, and their gross morphology recorded. All individual antral follicles (≥ 2 mm diam.) and corpora lutea were dissected free of extraneous tissue under a stereomicroscope and their diameters recorded to the nearest 0.5 mm. Dissection procedures, collection of follicular fluid, granulosa cell recovery and quantitation, isolation of theca interna as well as recovery and subsequent classification of oocytes (at $\times 40$ magnification) have all been described in detail elsewhere (McNatty *et al.*, 1983, 1984a). Some oocytes were also fixed in a sodium cacodylate buffer containing 3% glutaraldehyde (0.1 M, pH 7.35) for a more detailed histological analysis. After 1 h in fixative, the oocytes were rinsed twice in 0.1 M-sodium cacodylate buffer containing 1% (w/v) osmium tetroxide. After further rinsing in cacodylate buffer, the

oocytes were then stained with 2.0% (w/v) aqueous uranyl acetate for 40 min, dehydrated through a graded series of alcohols (30, 50, 70, 95 and 100%) and embedded in Spurr's Epoxy Resin. Light microscope sections were stained with 0.5% toluidine blue in 0.5% borax.

Classification of atresia in antral follicles. Current methodologies for classifying follicles into various stages of atresia before undertaking biochemical studies (Moor, Hay, Dott & Cran, 1978; Kruij & Dieleman, 1982; McNatty, 1982) were, in our hands, too unreliable for identifying those with a functionally active aromatase system in the granulosa cells and/or an LH-responsive theca interna (in terms of androstenedione synthesis). Accordingly, we developed a revised classification system which incorporated some of the criteria identified in the earlier studies. To assess the state of atresia of the various follicles, the following factors were considered: the presence or absence of thecal blood capillaries when the intact (but cleanly dissected) follicles were observed at $\times 10$ magnification; the presence or absence of debris in follicular fluid; the presence and status of the oocyte (healthy, degenerate or absent); the total number of granulosa cells expressed as a percentage of the maximum number of cells observed in a follicle of that size (McNatty *et al.*, 1984a); and the colour (or appearance) of the theca interna (red, pink or white). When these variables were collectively subjected to hierarchical cluster analysis (Genstat, 1981), they separated into four groups termed Grades 1, 2a, 2b and 3, with a similarity coefficient within clusters of 0.85. Grade 1 contained follicles with: a vascularized theca interna which was red, pink, or white, no debris in follicular fluid, $>75\%$ of the maximum number of granulosa cells for a given follicle size and a healthy-looking oocyte. Grade 2a contained follicles with: a vascularized red to pink theca interna, debris in the follicular fluid, $<75\%$ of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. Grade 2b contained follicles with: an avascular white theca interna, no debris in the follicular fluid, $<75\%$ of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate oocyte or no oocyte. Grade 3 contained follicles with a white avascular theca interna, debris in the follicular fluid, $<50\%$ of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. Grade 1, which was considered to contain the healthiest follicles, was arbitrarily divided into two subgroups (1a and 1b). All other characteristics being similar Grade 1a follicles were those with a vascularized theca interna which was red to pink and Grade 1b follicles had a vascularized theca interna which was white. Based on these 5 subgroups it was presumed that follicles would degenerate along two possible pathways namely: 1a \rightarrow 1b \rightarrow 2b \rightarrow 3 or 1a \rightarrow 2a \rightarrow 3.

Classification of follicles based on size and dominance. For each cow, follicles were classified as dominant, large (≥ 8 mm diam.), intermediate (5–7.5 mm diam.) or small (2–4.5 mm diam.). With the exception of Day +1, the dominant follicle was, for each cow, the Grade 1a follicle with the highest concentration of oestradiol in the follicular fluid and having granulosa cells with the highest aromatase activity. In 30/33 cows, one follicle in each animal was clearly identifiable as the dominant follicle. In the 3 remaining cows the above criteria fitted 2 follicles in each animal; in each of these animals both follicles were classified as dominant.

Dating the oestrous cycle. For each cow, the day of ovariectomy relative to the next presumed day of oestrus was calculated from a knowledge of the previous mean oestrous cycle length and the last date of oestrus. Subsequently, this was confirmed or modified by the histology of the corpus luteum (CL), the weight of CL, the binding characteristics of ^{125}I -labelled human chorionic gonadotrophin (hCG) to homogenized luteal tissue, the progesterone secretory characteristics of the dispersed luteal cells over 3 h *in vitro* and, in some instances, the LH and progesterone concentrations in peripheral plasma.

The histological assessment of CL age was especially useful in dating the oestrous cycle on Days -3 to -1 . The histological criteria were as follows: on Days -4 or -5 the luteal cells had granular cytoplasm and were of approximately equal size with large nuclei and well distributed chromatin.

The arterioles had large lumina. The general picture was of little connective tissue and a uniform appearance. On Day -3 occasional shrunken, darkly staining cells with pycnotic nuclei were scattered unevenly throughout the tissue. A few of the luteal cells were vacuolated. The lumina of the arterioles were smaller than on Day -4 or -5. In some places, the small arterioles were sclerotic. On Day -2 increasing numbers of shrunken cells with or without pycnotic nuclei were present and there was more variation in the size of the luteal cells. The granules in the luteal cell cytoplasm were less extensive and vacuolation was more pronounced. The arterioles were similar to those on Day -3 but a greater proportion had small lumina. More very small arterioles were evident than before, presumably because of an increase in the thickness of their walls. There was a general increase in the quantity of connective tissue which gave a marbled appearance throughout the CL. There were a few inflammatory cells and cell fragments. On Day -1 the number of shrunken cells was less than on Day -2. However, there was more variation in the size of the luteal cells which, overall, were smaller relative to those on Day -2. There was also a reduction in luteal cell number and more connective tissue was evident between the remaining cells. The arterioles had very small lumina and were numerous. There were small but scattered groups of inflammatory cells and debris. On Day 0, the extent of degeneration was much more obvious than on Day -1 and by Day +1 no luteal cells were apparent and most arterioles were sclerotic.

The mean \pm s.e.m. weight of the CL differed little between Days -5 and -1 (4.77 ± 0.14 g, $n = 22$). On Days 0 and +1 the mean \pm s.e.m. CL weight was 3.49 ± 0.25 g ($n = 7$) and 1.62 ± 0.2 g ($n = 4$), respectively.

From the criteria outlined by Henderson, Kieboom, McNatty, Lun & Heath (1984), the calculated equilibrium dissociation constants (K_d) and maximum binding capacities (B_{max}) for specific binding of 125 I-labelled hCG to homogenized luteal tissue did not differ for CL recovered from Day -5 to -1; over this period the respective mean \pm s.e.m. for B_{max} and K_d was 38.3 ± 3 fM/mg protein and 0.324 ± 0.027 nM ($n = 11$). On Day 0 these values were 16.9 ± 5.7 fM/mg protein and 0.239 ± 0.059 nM ($n = 4$) and on Day 1, 0 and 0 ($n = 2$).

Using the method of Henderson & Moon (1979) to isolate bovine luteal cells, it was found that the luteal progesterone output over 3 h in response to stimulation by LH (NIH-LH-B10; 100 ng/ml) varied with the day of the cycle. From Days -5 to -2 the mean output was relatively constant, i.e. 159 ± 24 ng/ 10^6 cells/3 h ($n = 6$), but from cells recovered on Days -1 and 0 the respective outputs were 30 ± 10 ($n = 4$) and 19 ± 9 ($n = 3$) ng/ 10^6 cells/3 h.

The mean \pm s.e.m. concentrations (ng/ml) of LH in plasma were 1.6 ± 0.06 ($n = 19$) on Day -5, 1.5 ± 0.07 (23) on Day -4, 1.6 ± 0.08 (20) on Day -3, 1.4 ± 0.1 (15) on Day -2, 1.7 ± 0.2 (10) on Day -1, 17 ± 6 (7) on Day -0 and 2.6 ± 0.4 (3) on Day +1. Since only one blood sample was recovered for LH analysis it was not possible to determine whether the ovaries on Day 0 were recovered during the ascending or descending limb of the preovulatory LH surge.

The mean \pm s.e.m. progesterone concentrations (ng/ml) in plasma were 5.1 ± 0.3 ($n = 19$) on Day -5, 4.6 ± 0.3 (23) on Day -4, 3.8 ± 0.3 (20) on Day -3, 2.6 ± 0.4 (15) on Day -2, 1.3 ± 0.3 (10) on Day -1, 0.9 ± 0.2 (7) on Day 0 and 0.3 ± 0.1 (3) on Day +1.

By assessment of all of the above criteria 30 of the 33 cows were judged to be at the day of oestrous cycle that would have been expected from the previous history, one at Day 0 instead of Day +1, one at Day 0 instead of -1 and one at Day -2 instead of Day 0. Overall, 2 cows were judged to be at Day -5 of the cycle, 3 at Day -4, 5 at Day -3, 5 at Day -2, 7 at Day -1, 7 at Day 0, and 4 at Day +1.

The granulosa cell aromatase assays. Granulosa cells from all individual follicles were collected into fresh Medium A. In most instances aromatase assays were performed on individual healthy (Grade 1a) follicles. On some occasions with small healthy follicles (<5 mm diam.) and on many occasions with atretic (Grades 1b, 2b and 3) follicles too few cells were available for individual assays and so several follicles were pooled. Care was taken to ensure that pools of cells originated from follicles of comparable diameter and stage of atresia. The cells were washed and resuspended

in Medium A containing sodium bicarbonate (0.85 g/l) so that the final cell concentration was $100\text{--}600 \times 10^3$ granulosa cells/ml; 0.5 ml aliquants of these cell suspensions were placed in 10×75 mm test tubes containing 0.5 ml of a solution of testosterone or androstenedione (for amounts added see below) in Medium A with sodium bicarbonate (0.85 g/l), gassed with 5% CO_2 in air, stoppered, and then incubated for 0, 0.5, 1, 2, 3, 4 or 6 h in a shaking water bath at 37°C . All assays were performed in duplicate. At the end of the incubation, the assay tubes were snap frozen to -70°C . Subsequently, the tubes were thawed, centrifuged and the supernatants assayed for oestradiol.

Three types of aromatase assays were performed. In one experiment the cells were incubated with androstenedione or testosterone at doses of 0, 3, 30, 300, 3000, 30 000 or 300 000 ng/ml to compare their respective potencies as substrates. In a second experiment cells from 40 individual healthy follicles (2.5–14.5 mm diam.) were subdivided into appropriate aliquants and incubated with 1000 ng testosterone for variable periods of time (see above) or for a fixed time (3 h) with variable amounts of testosterone. Preliminary studies revealed that the rate of oestradiol accumulation by granulosa cells exposed to different amounts of testosterone or androstenedione could be fitted to the Lineweaver–Burk or Eadie–Hofstee forms of the Michaelis–Menten equation with a correlation coefficient of >0.9 ($P < 0.01$). Consequently, the characteristics of aromatase enzyme activity in entire bovine cells were analysed using the enzyme kinetic programme of Crabbe (1982). In the third and most common experiment, the cells (from follicle pools or individual follicles) were incubated in testosterone at a final concentration of 1000 ng/ml for 3 h. Preliminary studies with 1000 ng testosterone substrate revealed that the rate of oestradiol formation was linear for the first 3 h for granulosa cells from atretic follicles and for most healthy follicles. However, in some healthy follicles, the rate of metabolism was not always linear after 2 h and for these follicles the results underestimated the true activity by up to 25%.

Theca interna perfusions. To determine the ability of theca interna to secrete androstenedione in the presence or absence of LH, samples of theca interna were perfused *in vitro* as described previously (McNatty *et al.*, 1984a). Briefly 25–50 mg theca interna tissue were recovered from the internal face of the follicle wall by microdissection, split into 4 fractions with 2 of these being placed into glass columns (4×0.7 cm, Econo-column: Bio-Rad Laboratories, CA, U.S.A.) containing 50 mg Sephadex G-25, pre-washed for 1 h with a perfusion medium consisting of sterile Krebs–Ringer–bicarbonate buffer containing 1% BSA (w/v) which was gassed continuously with 50% O_2 , 45% N_2 and 5% CO_2 . The 3rd thecal fraction was homogenized in 1 ml ethanol to determine the endogenous steroid content and the 4th was fixed for histological examination to test the purity of the tissue before perfusion. The thecal tissue on the columns was perfused at 37°C for 3 h at a flow-rate of 1.4 ml/min with 12 min fractions being collected. For 1 of the 2 theca fractions on the columns LH (200 ng/ml; NIAMDD-bLH-4) was added to the perfusion medium for 20 min after the theca had been perfused for 1 h. The other perfused theca was not exposed to LH and served as an unstimulated control. At the end of the 3-h period, the tissues were fixed for histological examination and the medium from each tube frozen until assayed for androstenedione by a specific radioimmunoassay (RIA) (McNatty, Gibb, Dobson, Thurley & Findlay, 1981). The androstenedione output from theca was expressed as the cumulative output (ng) per 10 mg theca interna over the 3-h period during and after LH stimulation or after control media perfusion. Analysis of all thecae fixed for histological examination revealed that 70–100% of the cell population was theca interna: the major contaminants were theca externa, stroma and/or red blood cells and only a few granulosa cells (i.e. <5 per $7 \mu\text{m}$ section) were ever present.

Hormone assays. All steroids were measured using previously published RIA procedures (McNatty *et al.*, 1981, 1982). Androstenedione, testosterone and oestradiol were extracted from peripheral blood (10 ml) or ovarian venous blood (1 ml) with diethyl ether (2×5 vol) and then separated by the Lipidex 5000, or Sephadex LH-20 column chromatographic methods outlined elsewhere (McNatty *et al.*, 1981). Progesterone in peripheral plasma (1 ml) was measured using an RIA procedure (Thornycroft & Stone, 1972). In follicular fluid progesterone, androstenedione and

oestradiol were measured directly in diluted (10–100-fold with 0.1 M-phosphate-buffered saline, PBS, pH 7.2) aliquants of follicular fluid. Testosterone was measured in follicular fluid after it had been diluted (10–100-fold) with PBS and extracted twice with 5 volumes of diethyl ether.

Androstenedione in Krebs–Ringer solution (from the thecal columns) and oestradiol in the aromatase assay solutions were measured directly without extraction.

Details regarding the working dilutions and specifications of the progesterone (WA-26), androstenedione (WA-965), testosterone (WA-36) and oestradiol (WA-27) antisera are provided elsewhere (McNatty *et al.*, 1981, 1984b).

The detection limit of steroids in follicular fluid was 2 ng/ml. The detection limits of progesterone, androstenedione, testosterone and oestradiol in plasma were 200, 2, 1 and 1 pg/ml respectively. The intra- and inter-assay coefficients of variation for all the above steroid assays were <12%.

Statistical procedures. The criteria used to investigate the various stages of follicular atresia were subjected to cluster analysis using the single-linkage procedure outlined in Genstat (1981). With this method, the similarity coefficient between two clusters is the greatest between any two units, one in each cluster. All units start in separate clusters and the algorithm clusters those with similarity levels greater than some preset level (between 0 and 1).

When analysing the data on hormones in follicular fluid or plasma, they were first normalized by log transformation and then subjected to analysis of variance (ANOVA) followed by pair-wise comparisons of means using the error mean square of the ANOVA. The results from these studies were expressed as geometric means and 95% confidence limits.

Results

Hormones in peripheral and ovarian venous plasma

The concentrations (geometric means and 95% confidence limits) of androstenedione, testosterone and oestradiol in peripheral plasma and in the veins draining the ovary containing a dominant follicle or the contralateral ovary on different days of the oestrous cycle are summarized in Table 1. In general, the mean concentrations of steroids in ovarian venous blood exceeded those in peripheral plasma, but the range of values in the former was large and significant differences for all steroids were only observed on Day 0. With the exception of Day –3, the oestradiol concentrations in veins draining ovaries with a dominant follicle were always significantly greater than those in peripheral plasma ($P < 0.05$). With the exception of Day –1, the androstenedione concentrations in the veins draining contralateral ovaries were always significantly greater than in peripheral plasma ($P < 0.05$). Oestradiol concentrations from the dominant ovary were significantly greater than those from the contralateral ovary ($P < 0.05$) only on Day 0.

Ovarian weights and follicle numbers

The weights of ovaries with and without CL (means \pm s.e.m.) were 10.07 ± 0.44 g ($n = 31$) and 5.71 ± 0.25 g (33) respectively. The mean \pm s.e.m. number of follicles ≥ 2 mm diameter per cow was 52.4 ± 4.1 ($n = 33$ cows). The mean \pm s.e.m. number of follicles in ovaries with and without CL were 26.5 ± 2.3 (31) and 25.5 ± 2.2 (33) respectively.

Follicular health and atresia

The distribution of healthy ovarian follicles (Grade 1a) with respect to diameter and day of the oestrous cycle is shown in Text-fig. 1. On each day, there was at least 1 large (≥ 8 mm diam.) healthy follicle in each cow. On Days –5, –4, –3, –2, –1, 0 and +1, the chance that the largest follicle in the ovary was the dominant follicle was 50 (1/2 cows), 66.7 (2/3), 60.0 (3/5), 80 (4/5), 85.7 (6/7), 71.4

Table 1. Steroid concentrations in peripheral plasma, and in ovarian veins draining ovaries with a dominant follicle and from the contralateral ovaries

Plasma source	Day from oestrus	No. of cows	Plasma steroid concentrations (pg/ml)		
			Androstenedione	Testosterone	Oestradiol
Peripheral			7 ^{a,p} (2-29)	14 ^p (5-40)	15 ^a (5-43)
Ovary with dominant follicle*	-3	5	37 ^a (3-459)	27 ^p (18-42)	98 ^a (4-1939)
Contralateral ovary			464 ^{b,q} (45-4722)	142 ^a (37-556)	30 ^a (11-78)
Peripheral			20 ^{a,p} (5-74)	23 ^a (4-132)	19 ^a (6-62)
Ovary with dominant follicle*	-2	5	493 ^{b,q} (90-2724)	104 ^a (36-299)	144 ^b (20-706)
Contralateral ovary			178 ^b (45-2018)	110 ^a (27-441)	38 ^a (15-95)
Peripheral			11 ^a (2-144)	14 ^a (2-171)	18 ^a (16-21)
Ovary with dominant follicle*	-1	3	185 ^a (5-7044)	86 ^a (23-314)	164 ^b (19-1380)
Contralateral ovary			118 ^a (4-3790)	61 ^a (6-614)	106 ^b (8-1352)
Peripheral			10 ^{a,p} (4-27)	7 ^a (2-23)	14 ^{a,p} (7-31)
Ovary with dominant follicle*	0	5	191 ^{b,q} (31-1164)	94 ^b (34-284)	1326 ^{b,q} (270-6568)
Contralateral ovary			86 ^{c,q} (38-240)	60 ^b (17-209)	162 ^{b,q} (46-1635)

Values are geometric means with 95% confidence limits in parentheses.

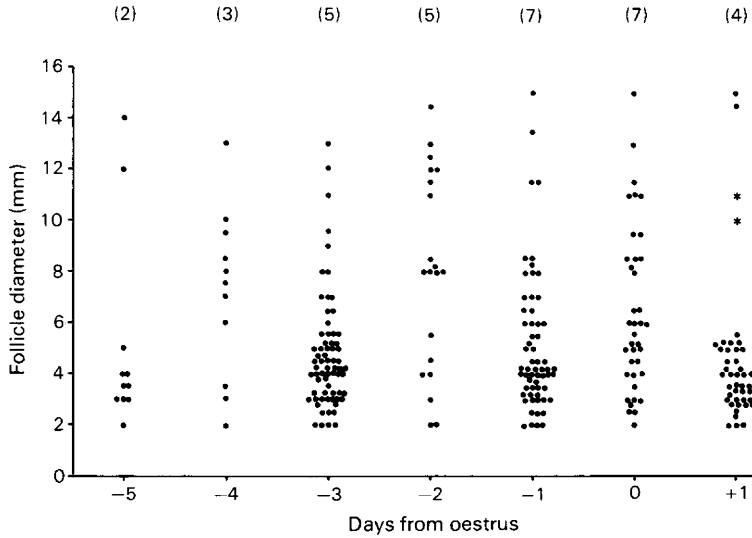
Numbers in columns for each day from oestrus with a different alphabetical superscript are significantly different from one another: ^{a,b,c} $P < 0.05$; ^{p,q} $P < 0.01$ (analysis of variance).

* Dominant refers to the Grade 1a (healthy) follicles in each cow with the highest concentration of oestradiol in follicular fluid and with granulosa cells having the highest level of extant aromatase activity.

(5/7) and 100% (4/4). The mean \pm s.e.m. number of healthy follicles (≥ 2 mm diam.) per cow was 7.6 ± 1.0 ; this represented $14.9 \pm 1.6\%$ of the follicle population.

Most (i.e. $85.1 \pm 1.5\%$) antral follicles (≥ 2 mm diam.) were atretic (i.e. Grades 1b, 2a, 2b and 3). The proportions of large (≥ 8 mm diam.), intermediate (5-7.5 mm diam.) or small follicles (2-4.5 mm diam.) in atresia did not vary with the day of the oestrous cycle. There was, however, an influence of follicular diameter on the distribution of follicles at each stage of atresia. The mean number of large follicles per cow was 2.4 ± 0.2 ; of this number $30 \pm 6\%$ per cow were atretic. Nearly all, 16/17 (94.1%) of these atretic follicles were in Grade 2a atresia; only one (5.9%) was in Grade 1b atresia. The mean number of intermediate-sized follicles per cow was 5.3 ± 0.6 ; of this number $67.3 \pm 7.4\%$ were atretic. Overall the distribution of intermediate-sized follicles at Grade 1b, 2a, 2b and 3 atresia was 37.0, 48.9, 5.4 and 8.7% respectively. The mean number of small follicles (2-4.5 mm diam.) per cow was 45.9 ± 4.5 ; of this number $92.9 \pm 1.4\%$ were atretic. Overall, the distribution of small follicles at Grades 1b, 2a, 2b and 3 atresia was 25.6, 22.7, 15.0 and 36.8%.

Of all Grade 2a, 2b or 3 follicles, 53% contained oocytes which appeared healthy, 13% contained oocytes which were clearly degenerate and from 34% of the follicles no oocyte was recovered. Healthy oocytes were not uncommon even in Grade 3 follicles which were severely deficient in granulosa cells.



Text-fig. 1. Total number of healthy follicles of different size with respect to day from oestrus (Day 0). Numbers in parentheses refer to number of cows studied on each day of the cycle.

* Haemorrhagic presumptive preovulatory follicles.

Follicular diameter and cell number

There was no effect of day of oestrous cycle on the diameter of the largest healthy (Grade 1a) follicle (Text-fig. 1), the diameter of the dominant follicle or their respective numbers of granulosa cells.

Steroids in follicular fluid

There was no difference for any steroid when the results from anaesthetized animals were compared with those from the slaughterhouse. Consequently, the results from these two groups of animals were pooled when appropriate. The concentrations (geometric means and 95% confidence limits) of progesterone, androstenedione, testosterone and oestradiol in the fluid of dominant, large, intermediate or small healthy (Grade 1a) follicles regardless of the day of oestrous cycle are shown in Table 2. As healthy follicles increased in size there were significant increases in the concentrations of progesterone and oestradiol ($P < 0.05$) and significant decreases in the concentrations of androstenedione and testosterone ($P < 0.01$). The dominant follicles differed from the large follicles in that they contained significantly higher concentrations of oestradiol ($P < 0.01$). When the data for each size of healthy follicle were analysed with respect to the day of the oestrous cycle, no significant changes in the concentration of progesterone, androstenedione or testosterone were observed. The only exception to this was noted for progesterone concentrations in dominant follicles on Day +1. On this day the progesterone concentrations in two follicles were 485 and 775 ng/ml; these concentrations were at least 16-fold greater than in all other dominant follicles on all other days of the cycle. There were no significant changes in oestradiol concentrations for small or intermediate-sized follicles with respect to day of cycle. For the large healthy follicles or dominant follicles, however (see Table 3), the mean concentrations of oestradiol on Day 0 were 2–10-fold higher than those on other days of the cycle although high oestradiol concentrations were also present in some follicles on Days -5, -4, -3, -2 and -1, but not on Day +1.

The concentrations (geometric means and 95% confidence limits) of progesterone, androstenedione, testosterone and oestradiol in the fluid of all atretic follicles (Grades 1b to 3) with respect to follicle diameter are summarized in Table 2. The data refer to follicles at Grades 1b, 2a, 2b or 3 of

Table 2. Steroid concentrations (geometric means and 95% confidence limits) in healthy and atretic bovine follicles

Follicle type or diameter (mm)	No. of follicles	Steroid (ng/ml)			
		Progesterone	Androstenedione	Testosterone	Oestradiol
Healthy					
Dominant*	32	30 ^{a,p} (24-39)	11 ^{a,p} (9-15)	17 ^{a,p} (11-27)	60 ^{a,p} (35-105)
≥8	56	27 ^{a,p} (22-33)	10 ^{a,p} (8-12)	14 ^{a,p} (10-19)	27 ^{b,q} (17-42)
5-7.5	45	20 ^{b,q} (16-23)	20 ^{b,p} (15-25)	31 ^{b,q} (23-42)	7 ^{c,r} (5-9)
2-4.5	77	15 ^{c,q} (13-18)	34 ^{c,q} (27-43)	59 ^{c,r} (47-74)	4 ^{d,r} (3-4)
Atretic					
≥8	17	176 ^{a,p} (100-305)	9 ^{a,p} (5-15)	15 ^{a,p} (6-38)	2 ^a (2-2)
5-7.5	73	33 ^{b,q} (25-45)	17 ^{b,p} (14-21)	23 ^{a,p} (18-30)	3 ^a (2-3)
2-4.5	707	30 ^{b,q} (28-33)	35 ^{c,q} (32-37)	42 ^{b,q} (38-46)	3 ^a (2-3)

Numbers (in columns) for either healthy or atretic follicles with a different alphabetical superscript are significantly different from one another: ^{a,b,c,d} $P < 0.05$; ^{p,q,r} $P < 0.01$ (analysis of variance).

* A dominant follicle was the Grade 1a follicle in each cow with the highest concentration of oestradiol in follicular fluid, and with the granulosa cells having the highest level of extant aromatase activity.

Table 3. Oestradiol concentrations (ng/ml) in dominant* or large healthy (≥8 mm diam.) follicles during the bovine oestrous cycle

Follicle type or diameter	Days from oestrus (Day 0 = oestrus)					
	-5 and -4	-3	-2	-1	0	+1
Dominant						
Oestradiol conc.	44 ^a (13-141)	30 ^a (3-324)	21 ^a (4-129)	83 ^{a,b} (37-185)	256 ^b (87-750)	20
No. of follicles	5	5	5	8	7	2
≥8 mm						
Oestradiol conc.	10 ^a (3-32)	16 ^a (5-60)	15 ^a (7-32)	57 ^{a,b} (27-123)	104 ^b (24-455)	20
No. of follicles	8	9	16	11	10	2

Values are geometric means with 95% confidence limits in parentheses.

Numbers (in rows) with a different alphabetical superscript are significantly different from one another, $P < 0.05$ (analysis of variance).

* A dominant follicle was the Grade 1a follicle in each cow with the highest concentration of oestradiol in follicular fluid and with granulosa cells having the highest level of extant aromatase activity.

atresia since no differences in steroid concentrations between these different grades of atresia were observed. Compared to small or intermediate sized atretic follicles, large atretic follicles contained the highest concentrations of progesterone, the lowest concentrations of androstenedione and testosterone and uniformly low or undetectable concentrations of oestradiol.

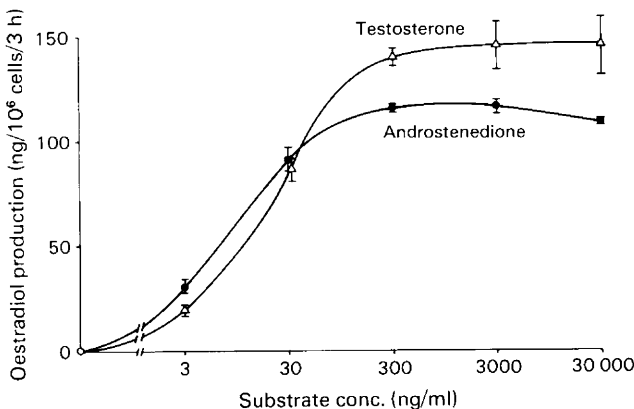
When the data for each size of atretic follicles were analysed with respect to day of the oestrous cycle, no significant changes were noted. Of the 17 large atretic follicles, 16 were assessed at Grade 2a atresia. When the steroid concentrations in these predominantly Grade 2a atretic follicles were compared to those in healthy or dominant follicles of comparable size, the atretic follicles contained the highest progesterone ($P < 0.01$) and the lowest oestradiol concentrations ($P < 0.01$) whereas the concentrations of androstenedione and testosterone were similar (see Table 2). Moreover, in small and intermediate atretic follicles the progesterone concentrations were significantly greater (both $P < 0.01$) than those in the respective healthy follicles (Table 2).

Aromatase activity in granulosa cells

The respective abilities of testosterone and androstenedione to be utilized by bovine granulosa cells for the synthesis of oestradiol are shown in Text-fig. 2. These androgens were equipotent as substrates for oestrogen biosynthesis.

The levels of aromatase activity in granulosa cells from healthy (Grade 1a), small (2–4.5 mm diam.), intermediate (5–7.5 mm diam.), large (≥ 8 mm diam.) and dominant follicles were 2.8 ± 0.4 (23 follicles), 12.5 ± 1.3 (33), 65.0 ± 11.2 (36) and 92.5 ± 15.6 (23) ng/ 10^6 cells/3 h respectively (intermediate *versus* small, $P < 0.01$; large *versus* intermediate, $P < 0.01$; dominant *versus* large, $P > 0.05$). Granulosa cells from all atretic follicles, regardless of grade of atresia or follicle size, contained little or no discernible aromatase activity (< 2 ng/ 10^6 cells/3 h, 195 follicles tested).

Aromatase activity in the granulosa cells was influenced by substrate concentration and incubation time. The characteristics of aromatase enzyme activity in cells from dominant follicles during the oestrous cycle are summarized in Table 4. Dominant follicles on Day -4 to -1 sometimes contained granulosa cells with similar aromatase enzyme characteristics to those at oestrus. Overall, the apparent K_m values in healthy but not dominant follicles (≥ 5 mm diam.; $n = 14$) were highly variable (i.e. undetectable or ranged from 40 to 448 nM) whereas the V_{max} values which ranged from 0 to 50 ng oestradiol/ 10^6 cells/3 h were consistently lower than those in cells from dominant follicles. The V_{max} and apparent K_m values in cells from a 10-mm presumptive preovulatory follicle on Day +1 were 0 nM and undetectable respectively. In cells from small healthy follicles (2–4.5 mm diam.) the apparent K_m values on Days -5 to 0 were undetectable or ranged from 621 to 1022 nM (9 follicles tested), whereas on Day +1 (2 follicles) they ranged from 27 to 37 nM. The V_{max} values in small healthy follicles on Days -5 to 0 (9 follicles) ranged from 0–4 ng/ 10^6 cells/3 h, whereas on Day +1 (2 follicles) they ranged from 12 to 26 ng/ 10^6 cells/3 h.



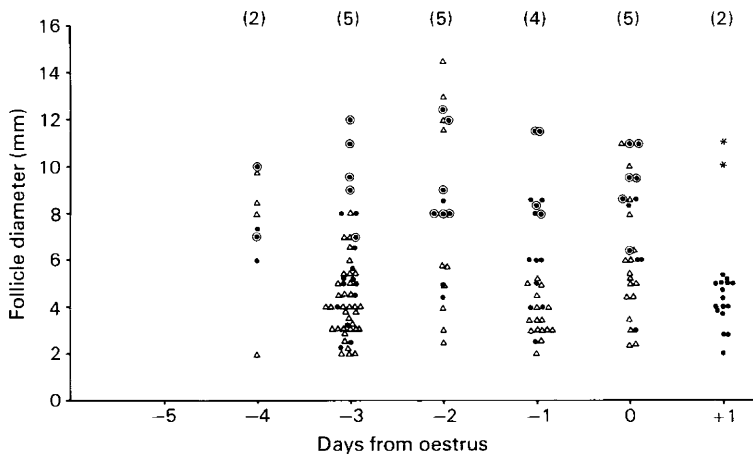
Text-fig. 2. Oestradiol output by bovine granulosa cells (250 000 cells/tube) from a pool of healthy large (≥ 8 mm diam.) follicles incubated with different concentrations of testosterone or androstenedione.

Table 4. Characteristics of aromatase enzyme activity in dispersed bovine granulosa cells from dominant* follicles during the oestrous cycle

	Days from oestrus (Day 0)		
	-4 and -3	-2 and -1	0
K_m (nM)	136 (118–156)	89 (18–132)	144 (74–209)
V_{max} (ng oestradiol/ 10^6 cells/3 h)	56 (21–296)	86 (38–120)	116 (109–244)
No. of follicles	3	4	5

Values are medians with ranges in parentheses.

* Dominant refers to the Grade 1a (healthy) follicles in each cow with the highest concentration of oestradiol in follicular fluid and with the highest level of extant aromatase activity.



Text-fig. 3. Scatter graph showing the relationship between diameter of healthy follicles and day of oestrous cycle relative to the day of oestrus (Day 0). ○, Dominant follicles with aromatase activity; ● and △, healthy but not dominant follicles containing granulosa cells with or without aromatase activity respectively; * haemorrhagic presumptive ovulatory follicles which were devoid of aromatase activity. Aromatase activity was assessed after incubation of granulosa cells with 1000 ng testosterone. The points refer to the total number of healthy ovarian follicles (≥ 2 mm diam.) from the number of cows indicated in parentheses.

Aromatase activity was examined in cells from all healthy follicles (using 1000 ng testosterone and a 3-h incubation time) from 23/33 cows with respect to day of the oestrous cycle (Text-fig. 3). Follicles with cells producing > 2 ng oestradiol/ 10^6 cells/3 h were recorded as having activity. On Days -5 to 0, the number of intermediate and large healthy follicles with aromatase activity was variable (0–100%). On Day +1, the recently ruptured or presumptive preovulatory follicles had no discernible aromatase activity (i.e. < 2 ng oestradiol/ 10^6 cells/3h) although all the small and intermediate-sized follicles had some activity (≥ 2 ng oestradiol/ 10^6 cells/3 h).

LH induced steroidogenesis in theca interna

Theca interna tissue from small, intermediate or large healthy follicles (Grade 1a, $n = 67$) responded to LH and produced 2.8–15-fold more ($P < 0.01$) androstenedione than did the

unstimulated tissues. The only exception to this was for LH-treated theca interna recovered from presumptive preovulatory follicles ($n = 2$) on Day +1; these tissues did not respond to LH and produced only trivial amounts of androstenedione (i.e. 3 and 9 ng/10 mg/3 h). For the other follicles that were 2–4.5 mm, 5–7.5 mm, ≥ 8 mm (on Days –5 to +1) or dominant (on Days –5 to 0), the respective androstenedione outputs by LH-stimulated thecae were 427 ± 101 (mean \pm s.e.m.; $n = 7$), 393 ± 47 (14), 425 ± 35 (23) and 453 ± 36 (23) ng/10 mg theca/3 h while the respective outputs from the unstimulated controls were 58 (2), 86 ± 13 (7), 96 ± 5 (19) and 87 ± 12 (15) ng/10 mg theca/3 h. These data show that there was no relationship between follicular diameter and thecal androgen output when the results were expressed per 10 mg tissue. It was also evident that the thecal androstenedione output from dominant follicles was similar to that from small, intermediate or large healthy follicles. When the data were analysed with respect to day from oestrus, no significant differences were observed. Even for the dominant follicle on Day 0, the level of LH-induced androstenedione biosynthesis was similar to that on other days of the oestrous cycle.

The LH-stimulated thecae from large atretic (Grade 2a) follicles ($n = 9$) produced significantly less androstenedione than did the controls (LH-treated theca, 27 ± 6 androstenedione/10 mg theca/3 h; control theca, 61 ± 8 ng/10 mg/3 h; $P < 0.01$). In contrast LH-stimulated theca interna from small and intermediate atretic (Grades 1b and 2a) follicles ($n = 10$) produced 1.2–8-fold more androstenedione than did their respective controls (LH-treated theca, 310 ± 32 ng/10 mg/3 h; controls, 68 ± 7 ng/10 mg/3 h; $P < 0.01$). Thecae internae from Grades 2b and 3 follicles were not studied because of the difficulties in identifying this tissue at these stages of atresia.

Relationship between LH-induced steroidogenesis in theca interna and extant aromatase activity in granulosa cells

The relationship between the LH-responsiveness of theca interna with respect to androstenedione production and the level of aromatase activity in granulosa cells from 57 individual healthy follicles (≥ 5 mm diam.) was examined. An LH-responsive theca interna was considered to be that producing more than twice the control outputs whereas granulosa cells having aromatase activity were those producing ≥ 2 ng oestradiol/ 10^6 cells/3 h. Most (i.e. 39/57 or 68%) of the healthy follicles contained measurable activity in both cell types although 18/57 (32%) contained an LH-responsive theca but no granulosa cell activity. No follicles were found with aromatase activity in granulosa cells in the absence of an LH-responsive theca or an absence of steroidogenic activity in both cell types.

Discussion

The results of the present study confirm and extend the notion that there is at least one large healthy follicle present in the ovaries of the cow on each day from Day –5 until ovulation, and that on each of these days there are 1–3 ovarian follicles ≥ 5 mm in each cow with an LH-responsive theca interna and extant aromatase activity in granulosa cells. The demonstration of highly variable aromatase activity in dominant follicles, highly variable concentrations of oestradiol in ovarian venous blood and follicular fluid and variable numbers of atretic follicles each day from Day –5 to Day 0 is consistent with the possibility that there is a continual turnover of follicles with a potential to ovulate (Matton *et al.*, 1981). Alternatively, and as a consequence of episodic gonadotrophin secretion (Hansel & Convey, 1983), it is possible that there are variable periodicities of activity and quiescence with respect to oestrogen biosynthesis in healthy follicles (≥ 5 mm diam.) which have a relatively slow turnover time (Scaramuzzi *et al.*, 1980).

In small healthy follicles (i.e. < 5 mm diam.) the presence of high androstenedione and testosterone, and low or undetectable concentrations of oestradiol in follicular fluid (Table 2), low or undetectable extant aromatase activity in the granulosa cells (Text-fig. 4; Table 4) and an LH-

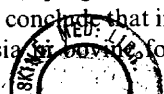
responsive theca provide strong evidence that early developing antral follicles develop a competence to synthesize androgens before appreciable aromatase activity is expressed in the granulosa cells. Presumably the increasing concentration of oestradiol at the expense of androgen as healthy follicles enlarge beyond 4.5 mm diameter (Table 2) is a direct consequence of an induction or activation of aromatase activity in granulosa cells.

In large healthy or dominant follicles, the LH responsiveness of theca interna (per unit mass of tissue) was similar to that in intermediate or small healthy follicles and did not differ with the day of the oestrous cycle. In dominant follicles, the level of androgen substrate required for aromatization by granulosa cells at 50% V_{max} can be derived from the apparent K_m value (i.e. 18–296 nM, Table 4). These amounts of androgen can readily be synthesized by LH-stimulated thecal tissues under in-vitro conditions (see 'Results' and McNatty *et al.*, 1984a). Therefore, the level of oestradiol biosynthesis by a dominant follicle *in vivo* is probably dependent upon the plasma concentration of LH and/or the LH pulse frequency as well as on the mass of theca interna which is known to increase with increasing follicular diameter (McNatty *et al.*, 1984a).

The follicle classification system described herein has provided a framework in which to examine some aspects of atresia. Of the intermediate and large follicles, between 1 and 5 per cow were assessed to be healthy (Grade 1a): 2–4 of these contained low or undetectable extant aromatase activity in their granulosa cells even though the theca from such follicles responded to LH by secreting androstenedione. Early atresia in intermediate or large follicles may be due to a failure to stimulate aromatase activity or to a loss of enzyme activity preceding any degenerative changes in the theca interna. In either case any treatment to enhance twin ovulations in cattle may need to sustain or activate aromatase activity in more than one of the large and/or intermediate-sized healthy follicles while these follicles await the preovulatory rise in LH secretion.

Of the small follicle population, ~93% were assessed to be atretic leaving on average only 3 healthy follicles per cow per day of cycle. Although the mean level of aromatase activity in small healthy follicles was indistinguishable from that in atretic follicles, it was clear that some individual follicles had enzyme activity whereas others did not. The substrate requirement in small follicles for half-maximum oestradiol biosynthesis (i.e. the apparent K_m value) was very high on Days –5 to 0 (521–1022 nM) whereas on Day +1 it was relatively low (27–37 nM). Furthermore, <25% of all small healthy follicles on Days –4 and 0 had measureable aromatase activity whereas all the small and intermediate-sized follicles on Day +1 had some activity (Text-fig. 3). A major difference between follicles on Day +1 compared to Days –4 to 0 is that all follicles on Day +1 would have experienced the preovulatory surge of LH and FSH (see Hansel & Convey, 1983, for review). Whilst the gonadotrophin surge may down-regulate aromatase activity in large follicles and initiate preovulatory luteinization (Dieleman, Kruip, Fontijne, de Jong & van der Weyden, 1983), it may concomitantly have increased the oestrogen-secreting potential of the small healthy follicles by altering the kinetic properties of the aromatizing enzyme system. The thecae from the small follicles on Day +1 still retained their ability to respond steroidogenically to LH *in vitro* (data not shown), but it is not known whether the increased incidence of aromatase activity in follicles on Day +1 was due to increased exposure to FSH, LH or androgen or any combination thereof.

Henderson *et al.* (1984) have shown that the theca interna from healthy and atretic follicles has specific LH/hCG receptors and that the binding characteristics of these receptors do not change with increasing atresia. However, as large follicles degenerate from Grade 1a to 1b or 2a, the thecae internae were no longer capable of secreting androgen or progesterone in response to LH (see 'Results'; Henderson *et al.*, 1984; McNatty *et al.*, 1984a). In conjunction with this loss of steroidogenesis in thecal tissue there were significantly higher progesterone concentrations in follicular fluid together with significantly lower (30-fold) oestradiol concentrations relative to those in dominant follicles (Table 2). Since small- and intermediate-sized as well as the large atretic follicles contained higher ($P < 0.01$) progesterone concentrations than healthy follicles of equivalent size, it seems reasonable to conclude that increased progesterone synthesis by granulosa cells is a common event during atresia in dominant follicles.



In contrast to the inability of LH to stimulate androgen synthesis in large atretic follicles, small- or intermediate-sized atretic follicles (Grades 1b and 2a) still retained a potential to synthesize androgen when stimulated by LH. Therefore, if all the healthy and many of the atretic follicles are capable of synthesizing androgen in response to LH, one could expect between 20 and 60 follicles (>2 mm diam.) per cow to secrete androgen in response to LH whereas only 1–3 would have the ability to metabolize the newly synthesized androgen to oestrogen. This finding may partly explain the extremely large variation in the concentrations of androstenedione in ovarian venous blood, the relatively high concentrations of androgen in small healthy and atretic follicles as well as the low concentrations of androgen in large healthy follicles (see also Wise *et al.*, 1982).

In conclusion our data suggest that most, if not all, healthy follicles (≥ 2 mm diam.) and many atretic follicles are biochemically competent to secrete androstenedione in response to LH whereas only 1–3 follicles (≥ 5 mm diam.) per cow have granulosa cells capable of metabolizing androstenedione to oestrogen. The aromatizing enzyme complex seems to be highly variable in activity and perhaps even unstable. The absence of aromatase activity in large or intermediate healthy follicles may indicate one of the earliest signs of atresia. Therefore, an understanding of the factors regulating granulosa cell aromatizing activity may help to provide new insights into methods of enhancing the ovulation rate in cattle.

We thank the National Pituitary Agency, Maryland, U.S.A. for the supply of bovine and ovine pituitary hormones; Mr R. M. Goodwin, Mr H. H. Gwilliam and Mr I. C. Scott for monitoring oestrous activity; Miss N. Hudson, Mrs K. Ball and Mrs J. McDiarmid for assistance with surgery, blood sampling, steroid radioimmunoassays and animal care; Mr P. Smith for preparing the oocytes for histology; Mrs K. Mason for preparing the enzyme kinetics programme; and Mrs P. Cattermole for typing the manuscript.

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Received 6 December 1983