Aust. J. Biol. Sci., 1987, **40**, 451–7

Morphometric Estimation of the Numbers of Granulosa Cells in Preovulatory Follicles of the Ewe

J. D. O'Shea, P. J. Wright and K. E. Davis

Departments of Veterinary Preclinical and Clinical Sciences,
University of Melbourne, Parkville, Vic. 3052.

Abstract

Several lines of evidence suggest that follicular granulosa cells give rise to the large luteal cells of the corpus luteum in the sheep. To further investigate this suggestion, numbers of granulosa cells in preovulatory follicles were estimated by morphometric methods for comparison with a previous estimate of numbers of large luteal cells ($9.6 \pm 0.9 \times 10^6$). Preovulatory follicles from five Corriedale ewes were obtained after synchronization of the oestrous cycle with the prostaglandin analogue cloprostenol. Morphometry was undertaken using light microscopy of plastic-embedded tissue sectioned at 1 μm. Mitotic index in the membrana granulosa was 0.05 ± s.e.m. 0.05%. Mean follicular diameter was 6.25 ± 0.25 mm and there were $7.68 \pm 0.53 \times 10^6$ granulosa cells per follicle. These results demonstrate a similarity between the number of granulosa cells per follicle and the number of large luteal cells per corpus luteum and thus support the hypothesis that large luteal cells are derived from granulosa cells.

Introduction

In sheep as in other ruminants, the corpus luteum (CL) contains two distinct types of steroidogenic luteal cells, large and small (O'Shea 1987). Several lines of evidence support the hypothesis that the large luteal cells are derived from follicular granulosa cells, and further that granulosa-derived cells undergo little if any mitosis after the time of ovulation (McClellan *et al.* 1975; O'Shea *et al.* 1980). This hypothesis would be strengthened if it could be demonstrated that numbers of granulosa cells in preovulatory follicles are similar to numbers of large luteal cells in the CL.

Numbers of large luteal cells in functional cyclical CL from Corriedale ewes have recently been estimated by ultrastructural morphometry (O'Shea *et al.* 1986). The purpose of the present study was to make a morphometric estimate of numbers of granulosa cells in preovulatory follicles from Corriedale ewes from the same flock, to permit a comparison between granulosa and large luteal cell numbers. For this purpose follicles were collected as close as possible to the time of anticipated ovulation.

Materials and Methods

Animals, Treatments, and Collection of Tissues

The Corriedale ewes used in this study were from the same flock as the ewes reported in a previous study of cell numbers in the CL (O'Shea *et al.* 1986). They were maintained on pasture supplemented with clover hay at the Veterinary Clinical Centre, Werribee, during a single mating season (April–May). All ewes were cyclic at the start of the experiment, as demonstrated by mating with a vasectomized ram fitted with a harness and crayon.

Ovulation and the development of preovulatory follicles were synchronized by the administration of two injections of the prostaglandin analogue cloprostenol ('Estrumate', I.C.I. Ltd., Macclesfield,

0004-9417/87/040451$02.00

Cheshire, England), 250 μg by intramuscular injection, 13 days apart. Based on preliminary observations on the timing of the preovulatory luteinizing hormone (LH) surge following injection of cloprostenol, blood samples for the measurement of LH concentration were collected at 3-hourly intervals from 22 to 58 h after the time of the second injection in all ewes, and up to 73 h in ewes from which CL were collected.

Twenty-eight ewes were included in this experiment. To confirm the occurrence of ovulation following the second injection of cloprostenol, nine ewes were maintained until 11 days after this injection (approximately 8 days after ovulation). Ovaries were then removed by laparotomy to detect CL, which were dissected from the ovaries, weighed and fixed in 10% formalin for histology. Laparotomy was performed on the remaining 19 ewes 60-65 h after the second injection of cloprostenol, and both ovaries removed and fixed whole in 10% formalin for examination of follicles.

Laparotomies were performed via a midline incision under pentobarbitone anaesthesia. Blood samples for LH assay were collected by jugular venepuncture and plasma was stored at −20°C.

Processing of Tissues, and Cell Counts

Luteal tissues were embedded in paraffin, sectioned at 5-7 μm, and stained with haematoxylin and eosin (HE).

Each follicle selected for morphometry was sliced at 90° to the surface of the ovary and a thick slice including approximately its central third was dehydrated in alcohol and embedded in JB-4 plastic embedding medium (Polysciences Inc., Warrington, Pennsylvania, U.S.A.). Serial sections were cut through the blocks so produced, using an Autocut 1150 Universal Microtome (Reichert-Jung, Heidelberg, West Germany). A regular pattern of sectioning was employed, in which one section at 50 μm in thickness alternated with two sections at 1 μm, and cutting continued until the diameter of the follicle had reached its maximum and commenced to decrease. Two 1-μm sections, 50 μm apart, were selected from the widest diameter of the follicle, and stained with HE. For each follicle these two sections were used for counts of mitotic figures and dead cells, and for morphometry.

Counts of mitotic figures and dead cells in the membrana granulosa were performed in a microscope fitted with an eyepiece graticule at a magnification of 320×. One hundred cells were counted at each of eight regions of the membrana granulosa, equally spaced around its margin (i.e. at 45° to one another), from each of two sections per follicle. Thus, percentages of dividing and dead cells were based on a total of 1600 cells per follicle.

Morphometric Methods

Light micrographs for morphometry were taken from each section at three magnifications, as follows: (*a*) 1 or 2 micrographs to include the whole follicle, at a negative magnification of 4×; (*b*) a series (7–13) of micrographs to include the whole of the membrana granulosa, at 16×; (*c*) a series of 8 micrographs of small areas of the membrana granulosa, including its entire thickness, at eight equally spaced points around its margin, at 100×. Prints were made in all cases at 5× the negative magnification (i.e. final magnifications of 20×, 80× and 500× respectively).

The cross-sectional area of each section of each follicle was measured, down to the basement membrane of the membrana granulosa, in whole follicle photographic prints, using an image analyser ('Videoplan', Carl Zeiss, Oberkochen, West Germany). Values obtained were corrected for print magnification (20×). The area occupied by the membrana granulosa was measured and corrected similarly, using prints at 80×, but excluding the cumulus oophorus if present in any micrographs. The total area of membrana granulosa included in the eight prints per section at 500× was also measured by image analysis.

From the measurements from micrographs at 20× and 80×, the volume of the membrana granulosa, excluding the cumulus, of each follicle was calculated assuming the follicles to be spherical, as follows:

True area of follicle (a) = mean measured area of follicle ÷ 400

True area of membrana granulosa (b) = mean measured area of membrana granulosa ÷ 6400

Area of antrum (c) = $a - b$

Radius of follicle (r_1) = $\sqrt{a/\pi}$

Radius of antrum (r_2) = $\sqrt{c/\pi}$

Volume of follicle (v_1) = $4/3\ \pi {r_1}^3$

Volume of antrum (v_2) = $4/3\ \pi {r_2}^3$

Volume of membrana granulosa = $v_1 - v_2$.

In each of the prints at 500×, counts of all granulosa cell nuclei, and point counts of all hits on granulosa cell nuclei (%), were made. Areas to be counted were defined by lines outlining the area measured as above: care was taken that the lines defining the antral and basal borders of the membrana granulosa corresponded precisely with the lines marking these borders in the equivalent prints at 80×, from which the total area of the membrana granulosa was measured. For each print 100 points were counted using a 21-bar, 42-point (M42) grid (Weibel 1979) reproduced on a transparent sheet and dropped repeatedly onto each micrograph in such a way that all depths of the membrana granulosa were represented equally. Thus, for each follicle the volume density (%) of granulosa cell nuclei was calculated from 1600 hits, and the total number of nuclei per unit area of membrana granulosa was calculated from counts of 16 areas of tissue. To correct for magnification, the measured area of these prints, at 500×, was divided by 250 000. For the purposes of calculating numbers of granulosa cells per unit volume of tissue, and per follicle, the following assumptions were made: (*a*) sections at 1 μm had no thickness; (*b*) each granulosa cell had a single nucleus; (*c*) granulosa cell nuclei were spherical; (*d*) all nuclei in the membrana granulosa belonged to granulosa cells; and (*e*) granulosa cell nuclei were uniform in size.

Numbers of nuclei (= cells) per unit volume (N_V) of granulosa tissue were calculated from the formula:

$$N_V = \frac{1}{\beta} \times \frac{{N_A}^{1.5}}{{V_V}^{0.5}} \quad \text{(formula 2.83, Weibel 1979),}$$

where β is a correction factor for nuclear shape (given a value of 1.382, representing a sphere). While divergence of nuclear shape from spherical would lead to some overestimation of N_V, the error would be small as long as shape approximates that of a sphere (Weibel 1979). N_A is the number of granulosa cell nuclei per unit area, and V_V is the volume density of granulosa cell nuclei. The total number of granulosa cells per follicle was then derived from N_V × volume of membrana granulosa.

To correct values of length, area and volume for tissue shrinkage or expansion during processing, and for any errors in photographic magnification, a linear correction factor of 1.12, relating measured values back to the original fixed tissues, was calculated and applied. For this, the diameters of follicles were measured at corresponding points in a stereoscopic microscope fitted with an eyepiece micrometer both before and after embedding (in transparent plastic medium), after sectioning, and after photographic enlargement.

LH Assay, and Definition of an LH Surge

Plasma LH levels were measured in a double-antibody radioimmunoassay (Wright *et al.* 1980) using NIH-LH-S18 (biopotency 1.03 × NIH-LH-S1) as standard. Hormone concentrations and assay quality control data were calculated by the method and computer program of Burger *et al.* (1972). Two assays were used in this study. Assay sensitivity was 0.56 ng/ml and the inter-assay coefficients of variation were 13.2, 18.0 and 16.6% for plasma pools of 9.3, 1.5 and 38.0 ng LH/ml respectively. The intra-assay coefficient of variation was <20% between 1.4 and 87.7 ng/ml.

Preovulatory surges of LH were defined on the basis of at least two consecutive plasma samples > 80 ng/ml LH.

Results

Evidence of Ovulation, and Selection of Follicles

Eight of nine ewes treated with cloprostenol and subject to laparotomy 11 days later had single (four ewes) or twin (four ewes) mature CL as confirmed by histology. These CL weighed 0.46 ± s.e.m. 0.04 g when a single, mean value was included for each pair of twin CL. The ninth ewe, which was presumed not to have ovulated, failed to show an LH surge up to 73 h from the second injection of cloprostenol, and had no detectable CL at laparotomy.

Four of 19 ewes in which laparotomy was performed 60–65 h after the second injection of cloprostenol failed to show an LH surge during the period of blood sampling. The remaining 15 ewes showed LH surges, first detected in plasma samples taken between 3.0 and 20.5 h before the collection of their ovaries. The largest follicle from each of five of these ewes, selected on the basis of maximum time from onset of LH surge to collection of ovaries, was used

for counts of mitoses and dead cells, and for morphometry. LH surges in these five ewes were first detected 18.00 ± 0.68 h (range 16.5 - 20.5 h) before collection of their ovaries.

Follicular Histology, Mitotic Index and Dead Cells

In all of the selected follicles the membrana granulosa showed a loosely-packed appearance (Fig. 1) consistent with preovulatory follicular histology. Granulosa cell nuclei appeared relatively uniform in size and rounded in shape, and no evidence was seen to suggest multinuclearity. Mitotic figures (Fig. 2) were rarely seen. The boundary between the membrana granulosa and theca interna was discernible in all follicles.

Mitotic index in the membrana granulosa was 0.05 ± 0.05%. Mitotic figures were also present in the theca interna (Fig. 3), being seen in both thecal and endothelial cells. Because of difficulty in identification of different cell types in the thecal interna, and in defining precisely the outer border of this layer, thecal mitoses were not quantitated; however, subjectively it appeared that mitoses were more numerous in the theca interna than in the membrana granulosa.

The incidence of dead cells in the membrana granulosa (Fig. 4), identified on the basis of nuclear pyknosis or karyorrhexis, was 0.05 ± 0.04%.

Morphometric Findings

The five follicles examined had a mean ± s.e.m. diameter of 6.25 ± 0.25 mm and a volume of 130.39 ± 14.58 mm^3. Volume of the membrana granulosa was 8.87 ± 0.92 mm^3, and there were 0.89 ± 0.08 × 10^6 granulosa cells per mm^3. The total number of granulosa cells per follicle was estimated to be 7.68 ± 0.53 × 10^6.

Discussion

The accurate selection of 'preovulatory' follicles which are actually destined to ovulate is clearly a central problem in any study in which follicles need to be removed before the time of ovulation. This is particularly true when follicles need to be processed intact for morphological studies, eliminating the possibility of functional studies on the follicular cells or of analysis of follicular fluid, which could provide additional evidence of preovulatory status (McNatty *et al.* 1982; Webb and England 1982).

In the present study, evidence that the follicles examined were destined to ovulate was based on the following points: (*a*) occurrence of a preovulatory-type LH surge in all ewes from which follicles were selected, coupled with the fact that LH surges were constantly followed by ovulation in ewes from which ovaries were not removed until 11 days after cloprostenol administration; (*b*) the largest follicle present immediately approaching or during oestrus, as studied here, normally does undergo ovulation (Smeaton and Robertson 1971; Bherer *et al.* 1977; Webb and England 1982); (*c*) retrospective confirmation that the histological structure of the membrana granulosa was consistent with preovulatory status, together with the very low incidence of cell death in the membrana granulosa, indicating the absence of atresia (Hay *et al.* 1976).

Cumming *et al.* (1971) have shown that ovulation normally occurs approximately 24 h after the onset of the preovulatory LH surge in the ewe. Hence it is likely that the follicles studied here were collected within $\simeq$ 4–10 h of their expected time of ovulation.

The present findings on absolute numbers of granulosa cells per preovulatory follicle are consistent with the data of McNatty *et al.* (1982), in which granulosa cell numbers were counted in a haemocytometer after mechanical dislodgement from the walls of follicles from Romney ewes treated with cloprostenol. These authors derived a formula to express the relationship between maximum number of recoverable granulosa cells and follicular diameter, and observed a maximum of $\simeq$ 10 × 10^6 granulosa cells in follicles of the largest sizes. Applica tion of this formula to a follicular diameter of 6.25 mm, as observed in the present study, would provide an estimate of 6.26 × 10^6 granulosa cells per follicle. Since this formula is

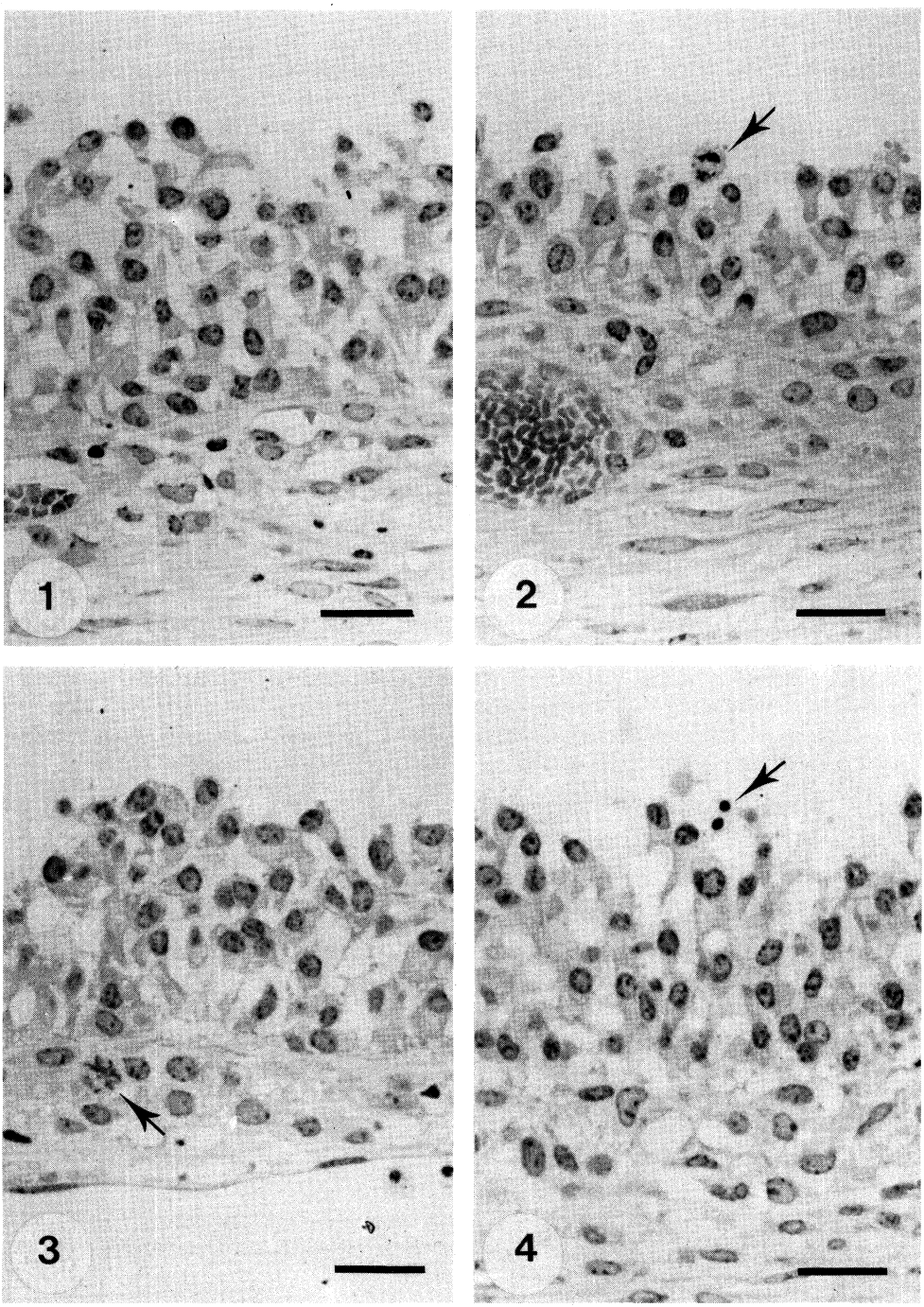

Fig. 1. Part of the wall of a preovulatory follicle, showing loose packing of the cells of the membrana granulosa. HE ×500 (bar = 25 μm).

Fig. 2. Follicle wall showing a mitotic figure (arrow) in the membrana granulosa. HE ×500 (bar = 25 μm).

Fig. 3. Follicle wall showing a mitotic figure (arrow) in the theca interna. HE ×500 (bar = 25 μm).

Fig. 4. Follicle wall showing a dead cell with karyorrhectic nucleus (arrow) at the antral margin of the membrana granulosa. HE ×500 (bar = 25 μm).

based on maximum *recoverable* granulosa cells, and may provide some underestimate of actual numbers, the similarity with the present morphometric estimate of $7.68 \pm 0.53 \times 10^6$ is remarkably close. A subsequent study from the same laboratory (McNatty *et al.* 1986) estimated granulosa cell numbers in preovulatory follicles from Merino ewes at 6.6×10^6, while Tsonis *et al.* (1984), using similar methodology, also found $6\text{–}7 \times 10^6$ granulosa cells in large follicles from luteal-phase Merino and Merino-cross ewes.

The only previous attempt to estimate granulosa cell numbers in 'preovulatory' ovine follicles by morphometry was that of Cahill and Mauléon (1980), using paraffin-section histology in Romanov and Ile-de-France ewes. Their study provided an estimate of 3.3×10^6 granulosa cells in the largest non-atretic follicles obtained at either day 0 or day 7 of the oestrous cycle. It is not clear why this earlier estimate was so much lower than that obtained in the present study, but breed differences, methodological differences and differences in the stage of follicular development could all have contributed.

When comparisons are made between granulosa cell numbers calculated here and numbers of large luteal cells as calculated previously ($9.6 \pm 0.9 \times 10^6$ — O'Shea *et al.* 1986), a similarity in absolute numbers is apparent. Such a similarity is consistent with the concept that granulosa cells give rise to large luteal cells without any major amount of intervening cell division. The present data, showing a very low mitotic index in the membrana granulosa of preovulatory follicles, together with earlier data showing that granulosa-derived cells in sheep appear to undergo little if any mitosis in the postovulatory period (McClellan *et al.* 1975; O'Shea *et al.* 1980), are also consistent with this concept of the origin of large luteal cells. Although estimated numbers of granulosa cells were in fact a little lower than the equivalent estimate of numbers of large luteal cells, this discrepancy could well be explained by experimental error coupled with inter-animal and inter-season variation. The existence of some, albeit low, levels of mitotic activity could also have contributed.

In relation to mitotic activity, doubling time (D.T.) for a population of cells can be calculated if the mitotic index (M.I.) and mitotic time (t) are known, from the formula:

$$\text{D.T.} = \frac{0.301\ t}{\log(1 + \text{M.I.})} \qquad \text{(Puck and Steffan 1963).}$$

Two estimates of mitotic time in ovine granulosa cells have been published, namely 0.43 h (Turnbull *et al.* 1977) and 1.38 h (Cahill and Mauléon 1980). Using these estimates, and the mitotic index of 0.05% reported here, doubling times for granulosa cells would be 596 h or 1914 h ($\simeq$ 24 - 80 days). These values suggest that, unless the mitotic index increases after ovulation, mitosis of granulosa-derived cells is unlikely to produce a major gain in cell numbers during the genesis of the CL.

In conclusion, the present estimate of absolute numbers of granulosa cells per preovulatory follicle is similar to an earlier estimate of large luteal cell numbers per CL: these findings are therefore consistent with an origin of large luteal cells from granulosa cells with little if any intervening mitosis.

Acknowledgments

Dr N. W. Bruce is thanked for valuable advice on morphometric methodology. We thank Ms Jenny Burden and Mr Phillip Smith for expert technical assistance. This work was supported by the Australian Meat Research Committee.

References

Bherer, J., Matton, P., and Dufour, J. J. (1977). Fate of the two largest follicles in the ewe after injection of gonadotrophins at two stages of the estrous cycle. *Proc. Soc. Exp. Biol. Med.* **154**, 412-14.

Burger, H. G., Lee, V. W. K., and Rennie, G. C. (1972). A generalized computer program for the treatment of data from competitive protein-binding assays including radioimmunoassays. *J. Lab. Clin. Med.* **80**, 302-12.

Cahill, L. P., and Mauléon, P. (1980). Influences of season, cycle and breed on follicular growth rates in sheep. *J. Reprod. Fert.* **58**, 321-8.

Cumming, I. A., Brown, J. M., Blockey, M. A. deB., Winfield, C. G., Baxter, R., and Goding, J. R. (1971). Constancy of interval between luteinizing hormone release and ovulation in the ewe. *J. Reprod. Fert.* **24**, 134-5.

Hay, M. F., Cran, D. G., and Moor, R. M. (1976). Structural changes occurring during atresia in sheep ovarian follicles. *Cell Tiss. Res.* **169**, 515-29.

McClellan, M. C., Diekman, M. A., Abel, J. H., and Niswender, G. D. (1975). Luteinizing hormone, progesterone and the morphological development of normal and superovulated corpora lutea in sheep. *Cell Tiss. Res.* **164**, 291-307.

McNatty, K. P., Gibb, M., Dobson, C., Ball, K., Coster, J., Heath, D., and Thurley, D. C. (1982). Preovulatory follicular development in sheep treated with PMSG and/or prostaglandin. *J. Reprod. Fert.* **65**, 111-23.

McNatty, K. P., Lun, S., Heath, D. A., Ball, K., Smith, P., Hudson, N. L., McDiarmid, J., Gibb, M., and Henderson, K. M. (1986). Differences in ovarian activity between Booroola × Merino ewes which were homozygous, heterozygous and non-carriers of a major gene influencing their ovulation rate. *J. Reprod. Fert.* **77**, 193-205.

O'Shea, J. D. (1987). Heterogeneous cell types in the corpus luteum of sheep, goats and cattle. *J. Reprod. Fert.* Suppl. **34**, 71-85.

O'Shea, J. D., Cran, D. G., and Hay, M. F. (1980). Fate of the theca interna following ovulation in the ewe. *Cell Tiss. Res.* **210**, 305-19.

O'Shea, J. D., Rodgers, R. J., and Wright, P. J. (1986). Cellular composition of the sheep corpus luteum in the mid- and late luteal phases of the oestrous cycle. *J. Reprod. Fert.* **76**, 685-91.

Puck, T. T., and Steffan, J. (1963). Life cycle analysis of mammalian cells. I. A method for localizing metabolic events within the life cycle, and its application to the action of colcemide and sublethal doses of X-irradiation. *Biophys. J.* **3**, 379-97.

Smeaton, T. C., and Robertson, H. A. (1971). Studies on the growth and atresia of Graafian follicles in the ovary of sheep. *J. Reprod. Fert.* **25**, 243-52.

Tsonis, C. G., Carson, R. S., and Findlay, J. K. (1984). Relationships between aromatase activity, follicular fluid oestradiol-17β and testosterone concentrations, and diameter and atresia of individual ovine follicles. *J. Reprod. Fert.* **72**, 153-63.

Turnbull, K. E., Braden, A. W. H., and Mattner, P. E. (1977). The pattern of follicular growth and atresia in the ovine ovary. *Aust. J. Biol. Sci.* **30**, 229-41.

Webb, R., and England, B. G. (1982). Identification of the ovulatory follicle in the ewe: associated changes in follicular size, thecal and granulosa cell luteinizing hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period. *Endocrinology* **110**, 873-81.

Weibel, E. R. (1979). 'Stereological Methods.' Volume 1, 'Practical Methods for Biological Morphometry.' (Academic Press: London.)

Wright, P. J., Geytenbeek, P. E., Clarke, I. J., and Findlay, J. K. (1980). Pituitary responsiveness to LH-RH, the occurrence of oestradiol-17β-induced LH-positive feedback and the resumption of oestrous cycles in ewes *post partum*. *J. Reprod. Fert.* **60**, 171-6.

Manuscript received 5 March 1987, accepted 12 August 1987