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The Pattern of Follicular Growth and Atresia in the Ovine Ovary

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Abstract

The ovaries of 21 2-year-old Merino ewes at various stages of the oestrous cycle were serially sectioned and all follicles >0·5 mm in diameter were counted and measured. The mean number of normal follicles >0·5 mm in diameter was 19·9 per ewe, of which 8·7 were >1 mm in diameter. The mean numbers of follicles >0·5 mm in diameter in 'early', 'advanced' and 'late' stages of atresia were 4·6, 9·0 and 12·3 respectively. Early atresia was rarely seen in follicles <1 mm in diameter; the greatest incidence was in follicles of diameter 1·5–2·5 mm.

Volumes of the granulosa, theca interna and antrum were calculated for all follicle sizes and the mitotic index of the granulosa and theca determined. Follicles were classified according to granulosa volume, each class having twice the volume of the preceding class. Estimates of the doubling time of the granulosa were made from the mitotic index and an estimate of mitotic time (0·43 h) obtained by use of colchicine on two other ewes. In all ewes, irrespective of day of oestrous cycle, the rate of growth was slow in follicles <0·4 mm in diameter; above this size it accelerated to a maximum (doubling time 26–30 h) in follicles 0·7–2 mm in diameter. It was estimated that, on average, a follicle takes about 5 days to grow from 0·5 to 2·2 mm diameter, and a further 4 days to reach 4·5 mm diameter. The number of follicles entering the rapid growth phase was estimated to be 3–4 per day. The present data do not support any particular 'wave' theory of follicular development, but indicate that follicles are growing or regressing asynchronously at any given time during the luteal stage of the oestrous cycle.

At 48 h after treatment of two ewes with PMS gonadotrophin (PMSG), the number of follicles >0·5 mm in diameter was increased, the greatest proportional increase being in those >3·5 mm in diameter. Relatively few follicles were undergoing early atresia. At 18 h after HCG treatment (given 48 h after PMSG to two ewes) all follicles >3·5 mm in diameter were undergoing luteinization and two-thirds of the follicles between 1 and 3·5 mm in diameter were undergoing early atresia.

Introduction

A number of workers over several decades have made histological studies of ovine Graafian follicles and have usually sought to relate their findings to the stage of the oestrous cycle at which the ovaries were removed (Grant 1934; Hutchinson and Robertson 1966; Brand and de Jong 1973). However, as each specimen represents only one point in time, it is difficult to obtain a true picture of the dynamics of follicular growth and regression. Using the mouse, this difficulty has been circumvented by pulse-labelling of the granulosa with tritiated thymidine and removing the ovaries at set intervals thereafter (Peters and Levy 1966; Pedersen 1970, 1972). This method was attempted with sheep but abandoned because of expense and practical difficulties. Instead, an attempt was made to obtain an understanding of the growth and regression of Graafian follicles from measurements of the volume and mitotic index of the granulosa of follicles of all sizes. From these data and a knowledge of the mitotic

time it is possible to make estimates of the time intervals involved in follicular growth (Leblond and Walker 1956).

Materials and Methods

Animals

A total of 27 medium-wool Peppin Merinos (2 years old) was used. Before slaughter all ewes exhibited regular oestrous periods as shown by markings by a harnessed, vasectomized ram.

Experimental Design

Twenty-one ewes were used to assess the number of normal and atretic ovarian follicles $> 0 \cdot 5$ mm in diameter.

The effects of gonadotrophins on follicular growth were studied in four other ewes. Two were injected subcutaneously with 1000 i.u. of pregnant mare serum gonadotrophin (PMSG) and the ovaries were removed 48 h later. The other two ewes were given 750 i.u. human chorionic gonadotrophin (HCG) intramuscularly 48 h after a subcutaneous injection of 600 i.u. of PMSG, and their ovaries were removed 18 h after the HCG injection. The gonadotrophin-treated ewes were between days 3 and 10 of the oestrous cycle at the time of injection.

In order to obtain an estimate of the time required for mitosis in the granulosa, the two remaining ewes were injected intravenously on day 3 of the oestrous cycle with 1 mg colchicine per kilogram live weight (Schinckel 1961). The dose was sufficient to stop all mitoses in metaphase. The ovary not containing a corpus luteum was removed, as a control, 2 h before the colchicine injection; the remaining ovary was removed 2 h after the injection.

Histological Methods

General

Ovaries removed at laparotomy were fixed whole in either Susa's or Serra's fixative, serially sectioned at 15 μm and stained with haematoxylin and eosin. Tracings of all follicles $> 0 \cdot 5$ mm in diameter and representative samples of smaller follicles were made with a projection microscope to enable identification for subsequent study at higher magnification.

Follicular dimensions and the diameters of the oocytes were measured with an ocular micrometer. Follicular diameter was taken as the mean of two measurements at right angles to each other on the section in which the area of the follicle was maximal. The third or vertical diameter was assumed to be of the same order. The basement membrane of the follicle was taken as the outer limits of the follicle. The volume of the granulosa was taken as the difference between the sphere bounded by the inner surface of the granulosa and the sphere bounded by the basement membrane. The volume of the theca interna was calculated from follicle diameter and thickness of the theca interna measured from the basement membrane of the follicle.

Classification of follicles

The follicle diameters of the classes shown in Table 1 were arbitrarily chosen. For estimation of the time intervals involved in follicular growth it is preferable to use classes based on doubling of the granulosa volume. A granulosa volume of 2×10^6 μm^3 was chosen as the starting point. Class 1 was defined as including follicles having a granulosa volume between $2 \cdot 0 \times 10^6$ and $4 \cdot 0 \times 10^6$ μm^3, class 2 follicles are those with a granulosa volume between $4 \cdot 0 \times 10^6$ and $8 \cdot 0 \times 10^6$ μm^3, and so on. Thus defined, there are 12 classes (see Tables 2 and 5).

Follicles were judged to be normal if there were no chromatin clumps or 'atretic bodies' (Knigge and Leathem 1956; Marion *et al.* 1968; Brand and de Jong 1973) associated with the granulosa. Follicles defined as undergoing 'early' atresia were those with a few atretic bodies on the inner edge of, or within, the granulosa. In such follicles, some abnormal and some normal mitoses were usually seen and there were also some pycnotic nuclei. Only normal mitoses were included in calculating the mitotic index. In follicles undergoing 'advanced' atresia there were no mitotic figures, the basement membrane was either partially or totally lacking, numerous atretic bodies were present, and a large proportion of the granulosa cells showed signs of disintegration. In follicles classified as being in 'late' atresia the cellular disintegration of the granulosa was either widespread or complete and the oocyte was either absent or undergoing disintegration.

Estimation of mitotic index

The number of mitotic figures in known volumes of granulosa and theca interna tissues was counted. Duplicate counts were made on two different sections and the mean number estimated. For follicles >0·5 mm in diameter a total of 100–200 mitoses per follicle was counted. The dimensions of individual granulosa and theca interna cells and their computed volumes were checked by a count of the number of cells occupying a known volume of tissue. The individual volume of the granulosa cells was found not to change appreciably with increase in follicle size, as has also been reported for Graafian follicles in pigs (Channing and Kammerman 1973). From these data, the proportion of cells undergoing mitosis at the time of fixation (the mitotic index) was calculated for both granulosa and theca interna in each follicle.

Estimation of rate of follicular growth

The time taken for a follicle to double the volume of its granulosa (i.e. to pass through one class) can be estimated from the mitotic index of the granulosa if the time that a cell spends in the process of mitosis is known. An esimate of this mitotic time was obtained from the use of the alkaloid, colchicine, which arrests all cells that enter mitosis, though it does not prevent completion of division in those cells that have already begun mitosis at the time of treatment (Hooper 1961; Puck and Steffen 1963). Thus, the mitotic index at the end of a period equal to mitotic time t should be the same as before treatment. At the end of $2t$ min, the mitotic index should be twice the pretreatment time. Thus the value of t can be calculated from the time between colchicine treatment and removal of the ovaries (2 h) and the increase in mitotic index. Under conditions of exponential growth (which appears to apply to the granulosa), the time taken for the number of cells to double is given by $(100t \times \ln 2)$/mitotic index, where $\ln 2 = 0{\cdot}693$ (Hoffman 1949).

Table 1. Mean number (±s.e.) of normal and atretic ovarian follicles per ewe

Follicles were classified according to diameter. Data were obtained from 21 Merino ewes

Type of follicle	Follicle diameter (mm)							
	0·50–0·75	0·75–1·00	1·00–1·25	1·25–1·50	1·50–2·50	2·50–3·50	>3·50	Total >0·50
Normal	7·7 ±1·3	3·5 ±0·5	2·4 ±0·4	2·0 ±0·4	3·0 ±0·7	0·85 ±0·2	0·45 ±0·1	19·9 ±2·7
Early atresia	0	0·1	0·7 ±0·2	0·55 ±0·2	3·0 ±0·7	0·2 ±0·1	0·1 —	4·7 ±0·9
Advanced atresia	0	1·6 ±0·4	2·0 ±0·5	2·2 ±0·4	2·7 ±0·7	0·35 ±0·1	0·1 —	8·8 ±1·6
Late atresia	8·0 ±1·3	3·5 ±0·6	0·65 ±0·2	0·15 —	0·05 —	0	0	12·3 ±1·6

Results

Follicle Populations during the Oestrous Cycle

The total number of normal and atretic follicles >0·5 mm in diameter present in the ovaries of the 21 untreated ewes is shown in Table 1. As each stage of the cycle was represented by one or two ewes only (and there were no data for days 0, 8 and 11), it is not possible to draw any definite conclusion about a correlation of follicle number with day of cycle. However, all stages of follicular growth and regression were present in the ovaries of each ewe. The number of follicles undergoing early atresia was much greater in the ewe examined on day 1 than in any of the other ewes (Fig. 1).

In all ewes, the mean number of normal follicles >0·5 mm in diameter (19·9 per ewe) was less than the mean number of atretic follicles in the same size range (25·95), indicating that the rate of follicular growth is somewhat faster than the rate of

regression. Early atresia was absent in Graafian follicles of less than about 1 mm diameter and the majority of follicles began to show early atresia at about 1·5–2·5 mm diameter.

Mensuration of normal follicles

The granulosa volume and mitotic index of the granulosa were determined for each of the 416 normal follicles selected from the ovaries of 18 of the original 21 ewes, i.e. two ewes from days 3 and 14 and one ewe from days 1, 2, 4, 5, 6, 7, 9, 10, 12, 13, 15 and 17 of the oestrous cycle. The diameter of the follicles ranged from 153 to 4930 μm (Table 2).

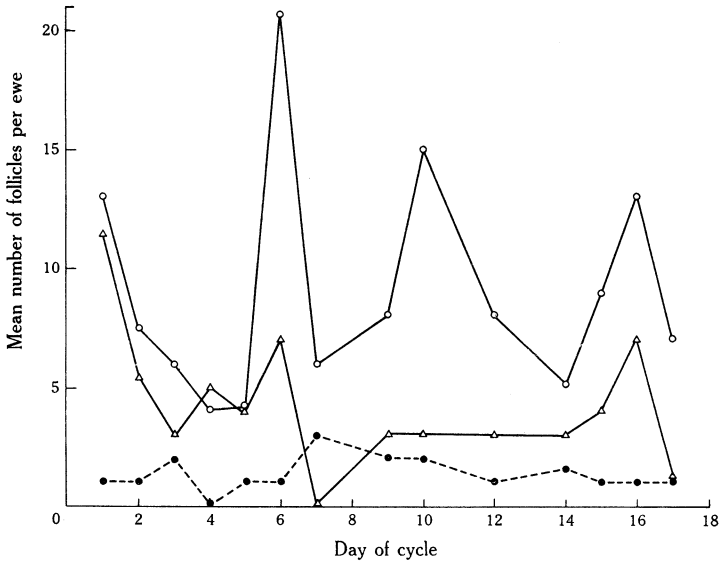

Fig. 1. Number of normal follicles >1·0 mm diameter (○) and >2·5 mm diameter (●) and number of follicles undergoing early atresia (△) in Merino ewes in relation to day of the oestrous cycle at which ovaries were removed.

For follicles of 150–700 μm diameter, there was a straight line relationship between log granulosa volume and log follicle diameter:

$$V = -5{\cdot}71 + 2{\cdot}75D,$$

where $V = \log_{10}$ granulosa volume (in 10^6 μm^3) and $D = \log_{10}$ follicular diameter (in μm). For follicles >700 μm in diameter the relationship was different but was also linear:

$$V = -2{\cdot}88 + 1{\cdot}76D.$$

Standard errors for the constants and slopes, respectively, were 0·05 and 0·02 (150–700 μm diameter follicles) and 0·07 and 0·02 (>700 μm diameter follicles).

The slope of the regression did not vary significantly for follicles <700 μm in diameter but did so for follicles >700 μm in diameter ($P < 0{\cdot}04$). However, with the latter follicles there was no trend with stage of cycle and the variation between ewes sampled on the same day of the oestrous cycle was as great as that between ewes

Table 2. Relation between follicle diameter, volume and mitotic index of the granulosa and of the theca interna, and oocyte diameter, relative fluid volume and rate of growth in normal ovarian follicles from 17 Merino ewes

Follicles classified according to granulosa volume. MI, mitotic index

Follicle class No.	Size range for class		No. of follicles in class	Mean diameter (μm)	Granulosa		Theca interna		Mean oocyte diameter ±s.e. (μm)	Follicle fluid volume[B] (%)	Estimated time in class (h)
	$10^{-6}\times$ Granulosa vol. (μm^3)	Diameter[A] (μm)			$10^{-6}\times$ Mean volume (μm^3)	Mean MI ±s.e.	$10^{-6}\times$ Mean volume (μm^3)	Mean MI ±s.e.			
1	2·0–4·0	153–197	18	189	3·24	0·13±0·02	3·1	0·11±0·03	73·6±1·8	—	230
2	4·0–8·0	198–253	35	225	5·91	0·13±0·02	5·0	0·08±0·02	83·2±1·1	—	230
3	8·0–16	254–326	40	290	11·9	0·16±0·02	10·9	0·10±0·02	92·9±1·2	6	186
4	16–32	327–419	71	367	23·7	0·24±0·02	19·6	0·17±0·02	100·7±0·9	9	124
5	32–64	420–540	59	480	46·4	0·46±0·03	43·9	0·39±0·03	106·0±1·0	20	65
6	64–128	541–690	42	609	88·6	0·75±0·05	85·6	0·55±0·04	109·7±1·1	26	40
7	128–256	691–1020	45	858	187	1·11±0·04	177	0·70±0·04	111·7±0·9	43	27
8	256–512	1021–1510	54	1223	360	1·12±0·03	324	0·55±0·04	115·1±1·0	62	27
9	512–1024	1511–2240	35	1752	660	0·98±0·05	601	0·36±0·04	120·6±1·3	77	30
10	1024–2048	2241–3325	10	2858	1582	0·63±0·10	1340	0·18±0·04	123·2±2·8	87	47
11	2048–4096	3326–4930	7	3722	2487	0·53±0·10	1606	0·19±0·06	123·3±3·2	91	56
											Total 1062

[A] Computed from the equations given in the text.

[B] Expressed as a percentage of the total volume of the follicle.

sampled on different days of the cycle. Accordingly, it seems that the variation with follicles >700 μm in diameter may have been due to differences in rates of accumulation of follicular fluid rather than differences in rates of proliferation of the granulosa. Furthermore, irrespective of day of cycle, the mitotic index of follicles 691–2240 μm in diameter (classes 7, 8 and 9) remained remarkably constant; the grand mean and standard error was $1{\cdot}06 \pm 0{\cdot}08$.

The largest follicle in the ovaries from the ewe sampled on day 17 (probably later pro-oestrus) was found not to fit the diameter–granulosa volume relationship. Presumably, it was undergoing normal rapid preovulatory enlargement and, accordingly, was omitted.

The 416 follicles referred to above were sorted according to granulosa volume into classes 1–11 and the mean follicle diameter, granulosa volume and mitotic index of the granulosa for each class was computed (Table 2). The mitotic index was low (0·13) in the small follicles but increased rapidly in those >0·4 mm in diameter. It reached a plateau of about 1·1 in follicles 0·7–1·5 mm in diameter, was slightly reduced in follicles 1·5–2·25 mm in diameter (0·98) and decreased to 0·5–0·6 in follicles >2·25 mm in diameter (Fig. 2).

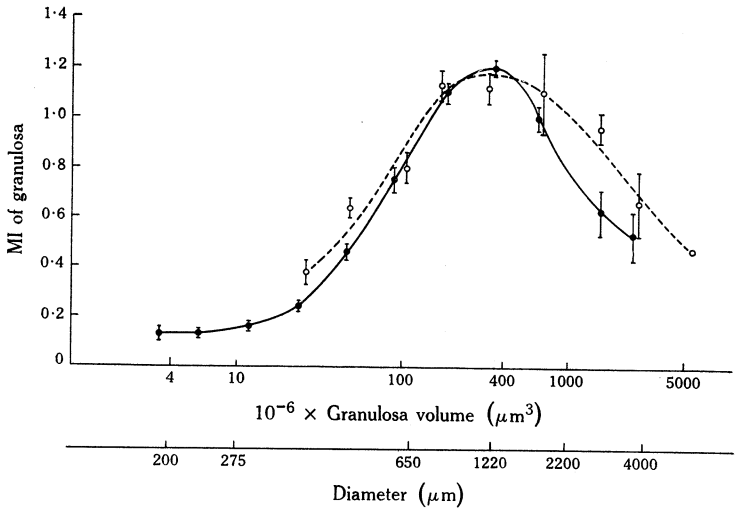

Fig. 2. Change in the mitotic index (MI) of the granulosa with growth of the follicle in untreated (●) and PMSG-treated (○) Merino ewes. Standard errors are shown by vertical bars.

The antrum was present in some 0·3-mm diameter follicles, was regularly seen in 0·4-mm diameter follicles and ultimately constituted 91% of the follicle volume in class-11 follicles (diameter 3326–4930 μm). The volume of the follicular fluid increases further in follicles about to ovulate: in the day-17 follicle referred to above, it constituted about 97% of the total volume of the follicle.

The theca interna appears to increase in volume at about the same rate as the granulosa up to about class 9 (Table 2). For larger follicles, difficulty was experienced in determining the outer limits of the theca interna.

Over the range of follicle sizes studied, the relationship between follicle diameter and oocyte diameter was found to be a bent hyperbola. Oocyte diameter continued

to increase slowly after the appearance of the antrum (follicular diameter 300–400 μm) and virtually ceased when the follicle reached a diameter of about 1500 μm.

Mensuration of atretic follicles

A random sample of 66 follicles classified as being in the early stage of atresia was measured (Table 3). Their diameter–granulosa volume relationship was described by the equation

$$V = -2{\cdot}13 + 1{\cdot}51D.$$

The standard errors of the constant and slope were 0·21 and 0·06 respectively. Statistical comparison of regression showed that the slope was significantly different ($P < 0{\cdot}001$) from that found for normal follicles >700 μm in diameter ($V = -2{\cdot}88 + 1{\cdot}76D$). Granulosa volume was smaller relative to total follicular volume in atretic than in normal follicles.

Table 3. Mean follicle diameter, volume and mitotic index of the granulosa, mitotic index of the theca interna and the relative fluid volume for various size classes of early and advanced atretic follicles

MI, mitotic index

Stage of atresia	Follicle class No.[A]	No. of follicles	Mean diameter (μm)	Granulosa		Theca interna		Follicle fluid volume[B] (%)
				10^{-6} × Mean vol. (μm^3)	Mean MI ±s.e.	10^{-6} × Mean vol. (μm^3)	Mean MI ±s.e.	
Early	7	2	986	225	0·58±0·16	167	0·21±0·15	55
	8	23	1450	396	0·60±0·06	324	0·16±0·02	75
	9	28	1959	707	0·37±0·04	571	0·12±0·01	82
	10	13	3056	1374	0·31	1068	0·10±0·02	91
Advanced	6	12	965	101	0	112	—	78
	7	17	1385	209	0	233	—	85
	8	26	1567	326	0	303	—	84
	9	5	2322	632	0	528	—	90
	10	2	3983	1193	0	1293	—	96

[A] Based on the granulosa volume as in normal follicle classes (see Table 2).
[B] Expressed as a percentage of the total volume of the follicle.

Another 62 follicles classified as being in the advanced stage of atresia were also measured (Table 3). In these follicles, there was a further decrease in the granulosa volume relative to antral volume. The atretic follicles were allotted to classes according to their granulosa volume, as was done for normal follicles (Table 2). This may be questionable because of the changing relations of granulosa volume and total volume as atresia progresses and because of the difficulty of accurately estimating granulosa volume in the advanced stage of atresia due to the disintegration of the granulosa. However, it does give some basis for comparison between stages and between normal and atretic follicles.

Estimation of the growth rate of follicles

In the ovaries (one per ewe) removed from the two ewes 2 h before colchicine treatment, there was a total of 14 non-atretic follicles in classes 7–9, whilst in the two ovaries removed after colchicine treatment there was a total of only 7 normal follicles

in the same size range. The mean diameter, granulosa volume and mitotic index of the granulosa for the 14 non-atretic follicles were 1031 μm, 248×10^6 μm^3 and $1 \cdot 14 \pm 0 \cdot 07$ respectively. The corresponding values for the 7 normal follicles were 1054 μm, 257×10^6 μm^3 and $5 \cdot 30 \pm 0 \cdot 37$ respectively. Thus the relative increase in mitotic index was 4·65 from which $t = 0 \cdot 43$ h (26 min). This estimate is similar to those for other tissues in other mammals, i.e. about 30 min (Lushbaugh 1956; Hooper 1961). Using the formula $100t$ (0·693)/mitotic index, and $t = 0 \cdot 43$ h, estimates of the time follicles spend in each size class were calculated (Table 2).

Table 4. The mean number (per ewe) of normal, early atretic, and luteinized follicles in ovaries removed from Merino ewes after treatment with PMSG or PMSG followed by HCG

Data were obtained from four ewes. Values in parentheses are those obtained for untreated sheep (see Table 1)

Treatment[A]	Type of follicle	Follicle diameter (mm)							
		0·50–0·75	0·75–1·00	1·00–1·25	1·25–1·50	1·50–2·50	2·50–3·50	3·50–5·00	>5·0
PMSG	Normal	13·0 (7·7)	9·5 (3·5)	3·5 (2·4)	3·5 (2·0)	5·0 (3·0)	4·0 (0·85)	4·0 (0·35)	1·0 (0·05)
	Early atretic	0	0 (0·1)	0 (0·7)	0 (0·55)	0 (3·0)	1·0 (0·2)	0 (0·1)	0
PMSG and HCG	Normal	11·0	3·0	4·5	1·0	1·5	0·5	0	0
	Luteinized	0	0	0	0	0	0·5	3·5	0·5
	Early atretic	0	1·0	1·5	3·0	10·0	0·5	0	0

[A] See Methods for details.

Effects of gonadotrophic stimulation

At 48 h after the PMSG treatment, the number of normal follicles >0·5 mm in diameter per ewe (43·5) was twice that found in untreated ewes (19·9, Table 4). The greatest proportional increase was in the follicles >2·5 mm in diameter. However, there were very few follicles undergoing early atresia. Measurements were made on all normal follicles >0·5 mm in diameter and after classification on the basis of granulosa volume as in Table 2, mean values for each class were calculated (Table 5). For follicles of 700–1620 μm diameter the log diameter–log granulosa volume relationship ($V = -3 \cdot 25 + 1 \cdot 94D$, standard error of constant and slope 0·26 and 0·09 respectively) was similar to that for follicles of 700–4500 μm diameter in untreated ewes ($V = -2 \cdot 85 + 1 \cdot 76D$; see above). In PMSG-treated ewes the relationship for follicles >1620 μm in diameter was $V = -1 \cdot 6 + 1 \cdot 30D$ (standard error of constant and slope 0·22 and 0·07 respectively) and this differed significantly ($P < 0 \cdot 05$) from that for untreated ewes.

The mitotic index of the ganulosa was significantly higher ($P < 0 \cdot 05$) in PMSG-treated ewes than in untreated ewes for follicles in classes 4 and 5 (diameter 327–540 μm) and for class-10 follicles (diameter 2241–3325 μm) (Fig. 2). For classes 5–8, the mitotic index of the theca interna was significantly lower ($P < 0 \cdot 05$) in PMSG-treated than in untreated ewes, but for follicles in classes 10 and 11 it was significantly higher ($P < 0 \cdot 01$).

In the ewes injected with HCG 48 h after the PMSG treatment the ovarian pattern was dramatically different from that found in ewes given PMSG only (Table 4). The

majority of follicles 1·25–3·5 mm in diameter were undergoing early atresia, but those >3·5 mm in diameter were undergoing enlargement and luteinization. In these latter follicles the eggs were in metaphase of the first meiotic division—as might be expected from Dzuik's (1965) data on the timing of meiosis in relation to HCG treatment in ewes. In the follicles undergoing early atresia and in the normal follicles >1·5 mm in diameter the germinal vesicle was still present in the oocytes.

Table 5. Mensuration of follicles in ovaries removed from two Merino ewes 48 h after PMSG treatment and from two ewes 18 h after HCG treatment

HCG was given 48 h after PMSG treatment

Follicle class No.	Treatment group	No. of follicles	Mean diameter (μm)	Granulosa		Theca interna	
				$10^{-6}\times$ Mean vol. (μm^3)	Mean MI ±s.e.	$10^{-6}\times$ Mean vol. (μm^3)	Mean MI ±s.e.
4	PMSG	10	354	26·3	0·38±0·05	19·9	0·16±0·04
	PMSG+HCG	11	417	26·5	0·24±0·01	17·9	0·13±0·04
5	PMSG	26	503	48·1	0·64±0·04	34·6	0·24±0·02
	PMSG+HCG	18	481	44·2	0·50±0·04	28·0	0·29±0·04
6	PMSG	13	679	107·5	0·79±0·06	62·4	0·27±0·05
	PMSG+HCG	16	643	90·3	0·88±0·04	59·8	0·42±0·03
7	PMSG	16	830	172·4	1·13±0·06	124·2	0·36±0·06
	PMSG+HCG	5	790	166·6	0·94±0·07	116·7	0·50±0·06
8	PMSG	14	1148	328·2	1·12±0·06	185·5	0·31±0·04
	PMSG+HCG	13	1223	350·0	0·98±0·04	171·2	0·43±0·07
9	PMSG	7	1950	679	1·11±0·16	433·5	0·39±0·06
	PMSG+HCG	3	1592	564	0·81±0·07	301·0	0·09±0·02
10	PMSG	12	3221	1580	0·96±0·06	1208	0·46±0·05
11	PMSG	6	4786	2631	0·66±0·13	2095	0·47±0·10
12	PMSG	1	7340	5581	0·47	5335	0·25

Measurements made on 24 normal and 8 luteinized follicles in the ewes treated with PMSG and HCG indicated that for normal follicles >700 μm in diameter the log diameter–log granulosa volume relationship ($V = -3·01+1·81D$) was similar to that in untreated ewes (see p. 232), but for luteinized follicles it was markedly different ($V = 0·96+0·74D$), the granulosa representing a greater proportion of the total volume in normal follicles. The mean dimensions of the 8 luteinized follicles were: diameter 4117 μm, granulosa volume 4492×10^6 μm^3. In the follicles from ewes treated with both PMSG and HCG the mitotic index of the granulosa was significantly lower than normal for classes 4, 5 and 6 ($P < 0·05$) and classes 7 and 8 ($P < 0·02$). For follicles in classes 4 and 5 the mitotic index was significantly lower in the ewes that received PMSG and HCG than in the ewes treated with PMSG alone ($P < 0·05$).

Discussion

The data show that the growth of ovine ovarian follicles prior to the final preovulatory enlargement follows a well defined pattern which, on the basis of changes in the mitotic index of the granulosa with change in size of follicle, bears a general similarity to that occurring in the rat (Lane and Davis 1939), guinea pig (Schmidt 1942) and cow (Rajakoski 1960). Irrespective of the stage of oestrous cycle, the growth rate was slow in follicles up to about 0·4 mm in diameter and then accelerated until it

reached a maximum in follicles of about 0·7 mm diameter. This fast rate was maintained until the follicles reached a diameter of about 2 mm and then declined by about 50%. The regularity of the growth pattern is indicated by the straight line relationships between log follicle diameter and log granulosa volume, and the change in the slope of the relationship at a follicle diameter of about 0·7 mm could be associated with a change in the rate of accumulation of follicular fluid relative to the rate of granulosa growth.

For the 21 Merino ewes in the main study, the ovulation rate was 1·0 and the mean number of normal (non-atretic) follicles >0·5 mm in diameter per ewe was 19·9 of which 8·7 (44%) were >1 mm in diameter. Brand and de Jong (1973) found in 33 1½-year-old cyclic Texel ewes of ovulation rate 1·7, that there were 35 normal follicles >0·5 mm in diameter per ewe, of which 13 (37%) were >1 mm in diameter. Conjointly, these separate observations suggest the possibility that there may be a positive correlation between the number of small developing ovarian follicles and ovulation rate in ewes.

In Merino ewes, as in Texel ewes (Brand and de Jong 1973), signs of early atresia were rarely seen in ovarian follicles <1 mm in diameter but approximately two-thirds of the follicles of diameter 1·5–2·5 mm exhibited signs of early atresia. As atresia progressed, the granulosa volume decreased more rapidly than the fluid volume. The early stage of atresia could occupy approximately 2 days for there were few follicles of this type in the ovaries of ewes 48 h after PMSG treatment. The relative numbers of early, advanced and late atretic follicles (Table 1) indicate that the two latter stages of atresia occupy approximately 4 and 6 days respectively.

For a variety of mammalian tissues the mitotic time is about 30 min (Lushbaugh 1956; Hooper 1961). In the present study, that for granulosa cells was estimated to be about 26 min. Using the latter value, it is apparent that, in ewes, ovarian follicles take, on average, about 124 h to grow from 0·55 to 2·1 mm diameter (i.e. through classes 6–9) and approximately another 103 h (through classes 10 and 11) to reach about 4·5 mm diameter. As the highest mitotic index seen in follicles of classes 10 and 11 was 0·84, the minimum time for follicles to pass through classes 10 and 11 would be 70 h. A diameter of 4·5–5·0 mm appears to be the maximum size to which follicles normally grow in the absence of a pre-ovulatory gonadotrophic stimulus.*

From the estimates obtained, it was concluded that the follicle ovulating at the end of any particular cycle would have had a diameter of <0·5 mm on about day 6 or 7 of that cycle. An estimate of the number of follicles entering the rapid growth phase (class 5) per day may be obtained by dividing the mean number of follicles 0·5–1·0 mm in diameter per ewe (as there are virtually no losses from atresia in this size range, see Table 1) by the estimate of the time taken for follicles to grow through this range (about 3 days, Table 2). Thus it was concluded, in the Merino ewes studied, that 3–4 follicles per day entered the rapid growth phase. Pedersen (1972), using the pulse-labelling technique, estimated that in mature mice 10–20 follicles per day begin rapid growth. He has also provided estimates of the time intervals involved in follicular growth in the mouse (Pedersen 1970).

PMSG treatment of the ewes appeared to result in a doubling of the number of normal follicles >0·5 mm in diameter within 48 h. The increase could be attributed to an increase in the number of follicles entering the rapid growth phase (0·5–1·0 mm

* These diameters relate to fixed ovaries.

diameter) and to a lack of atresia in follicles >1 mm in diameter. In hamsters, Greenwald (1962) found that PMSG treatment prevented follicular atresia. In the present study, when HCG treatment was given 48 h after PMSG most of the ovarian follicles 1·25–3·5 mm in diameter became atretic and all the follicles >3·5 mm in diameter underwent luteinization. HCG treatment has also been reported to induce widespread atresia of growing follicles in rats (Selye *et al.* 1933) and guinea pigs (Reed and Hounslow 1971). In ewes the high incidence of atresia in follicles 1·25–3·5 mm in diameter following HCG treatment would appear to be analogous to the 'wave' of follicular atresia reported in a number of species at about the time of ovulation and thus about 1 day after the LH surge (Engle 1927; Myers *et al.* 1936; Block 1951; Mandl and Zücherman 1952; Greenwald 1961; Adams *et al.* 1966). In some species atresia occurring around the time of ovulation results in the demise of virtually all the medium and large follicles that do not ovulate. This, rather than variation in either follicular growth rate or the number of follicles entering the rapid growth phase, appears to be responsible for the appearance of a wave of follicular growth beginning early in the cycle (e.g. Mandl and Zückerman 1952).

In the ewe, the number of follicular growth waves during the oestrous cycle has been variously reported to be one (Hutchinson and Robertson 1966), two (Brand and de Jong 1973) or 3–4 (Smeaton and Robertson 1971). The latter workers marked (with carbon) the largest follicle in the ovaries of a number of ewes and found that only follicles marked on the last day of the cycle ovulated; large follicles marked earlier in the cycle regressed before ovulation. The present data do not support any particular wave theory; instead, they indicate that follicles are growing or regressing asynchronously at any given time during the luteal stage of the oestrous cycle.

It is not easy to reconcile this concept with the report of a number of peaks in oestradiol-17β secretion during the luteal phase of the cycle, i.e. on days 3–4 (Cox *et al.* 1971), 6–9 and 11–15 (Mattner and Braden 1972). The question arises as to whether or not these peaks of oestrogen secretion occur because some follicles reach a certain morphological development on appropriate days of the cycle or are occasioned by fluctuations in hormonal levels that result in oestrogen being secreted by the largest available follicle (Holst *et al.* 1972; Moor 1973). Such unanswered questions serve to emphasize that, despite intensive morphological and endocrinological investigations over several decades, a workable theory for the control of growth and atresia in the ovarian follicle and the functions of FSH and LH in this respect, has not been produced (Greenwald 1972; Schwartz *et al.* 1973).

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