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Gonadotropin Receptors of the Ovine Ovarian Follicle during Follicular Growth and Atresia[1]

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ABSTRACT

The binding of [^{125}I]-labeled human follicle stimulating hormone ([^{125}I]-FSH) and chorionic gonadotropin ([^{125}I]-hCG) to intact ovine follicles was studied *in vitro* as a function of follicular diameter and stage of morphological atresia.

Histological confirmation of the atretic classification was established and the incidence of atresia in those follicles studied was variable and directly related to follicular diameter ($P<0.01$). The binding of [^{125}I]-hFSH and [^{125}I]-hCG to theca was relatively constant when compared to the change in granulosa binding that was associated with increased follicular diameter or stage of atresia. When studied without regard to the stage of atresia, the binding of [^{125}I]-hFSH to granulosa cells decreased and that of [^{125}I]-hCG increased with increased follicular diameter. These changes were thought to reflect changes in the relative incidence of atresia within each size group rather than decreased binding *per se*. Subsequent analysis of [^{125}I]-labeled gonadotropin binding to granulosa cells as a function of both follicular diameter and stage of atresia simultaneously indicated that the extent of [^{125}I]-hFSH binding was determined solely by stage of atresia rather than follicular diameter ($P<0.05$). Conversely, while [^{125}I]-hCG binding was decreased by increased atresia, the overall extent of binding was determined by follicular diameter ($P<0.01$).

It is concluded that macroscopic assessment of follicular atresia in ovarian follicular populations is directly related to follicular diameter. In addition, the ability of granulosa cells to bind [^{125}I]-labeled gonadotropins *in vitro* varies as a function of follicular diameter and stage of atresia.

INTRODUCTION

Once initiated, follicular growth and maturation are continuous until either atretic degeneration or ovulation occurs (Peters, 1969; Turnbull et al., 1977). Successful follicular maturation is dependent upon sequential development of follicular responsiveness initially to estrogen and FSH and later LH (Zeleznik et al., 1974; Richards et al., 1976; Rajaniemi et al., 1977). Atresia may then be related to a decrease in follicular sensitivity to gonadotropins (Richards and Midgley, 1976).

In order to define the pattern of gonadotropin sensitivity associated with follicular growth and atresia, the binding of [^{125}I]-labeled gonadotropins to ovine follicles *in vitro* was studied as a function both of follicular diameter and stage of morphological atresia.

MATERIALS AND METHODS

Collection and Processing of Tissues

Ovaries were obtained from adult ewes within 15

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min of death and transported to the laboratory in saline (NaCl 0.154 M, Kanamycin 50 U/ml) at ambient temperature. During dissection of follicles from surrounding stromal tissue, ovaries and isolated follicles were maintained in incubation medium (Medium 199, HEPES 5 mM, calf serum 4% v/v, Kanamycin 50 U/ml, pH 7.4) equilibrated with a gas phase of 95% O_2:5% CO_2 (v/v).

For histological assessment, whole follicles or portions of theca were immersed in Bouin's fixative for 24 h at 4°C prior to preparation of paraffin sections (5 μm) and staining with haematoxylin, eosin and celestin blue.

Classification of Follicles

Prior to incubation, isolated follicles were grouped according to both follicular diameter and stage of morphological atresia based on descriptions of atretic follicles by Hay, Moor and others (Hay et al., 1976; Moor et al., 1978). Follicular diameter was determined by direct comparison of follicles with a scale graduated at intervals of 1 mm. Follicles were assigned to 1 of 5 stages of morphological atresia as judged by continuity of the membrana granulosa, the degree of thecal vascularization and the presence of a cumulus-oocyte unit when examined using transmitted light at 8 X magnification. Follicles of Class I were nonatretic while Class V were terminally atretic (Figs. 1a–e).

Preparation of [^{125}I]-Labeled Gonadotropins

Human FSH (hFSH; NIH-FSH-HS-1, 4990 IU/mg) and human chorionic gonadotropin (hCG; CR119, 11600 IU/mg) were iodinated using lactoperoxidase/H_2O_2 (Miyachi et al., 1972) and chloramine-T (Kammerman and Canfield, 1972), respectively. In each instance products were purified by gel chromatography (Biogel P60, 25 cm X 1 cm) and a specific activity of 23–35 μCi/μg was attained.

The binding of eluted fractions of [^{125}I]-hFSH to a preparation of bovine testicular membranes (Cheng, 1975) or [^{125}I]-hCG to a crude homogenate of rat ovaries was assessed prior to experimentation. Those fractions exhibiting maximal specific binding were employed in follicular binding studies.

Incubation

Groups of follicles were placed in siliconized glass vessels containing incubation medium to which [^{125}I]-labeled gonadotropin (10^{-10} M, 2 X 10^5 cpm/ml) had been added and incubated at 37°C under an atmosphere of 95% O_2:5% CO_2 with constant mechanical agitation. Nonspecific binding was determined in the presence of unlabeled gonadotropin at a concentration of 200 IU/ml (FSH; Primantron or hCG; Pregnyl). Specific binding was then calculated by subtraction of counts bound in the presence of excess

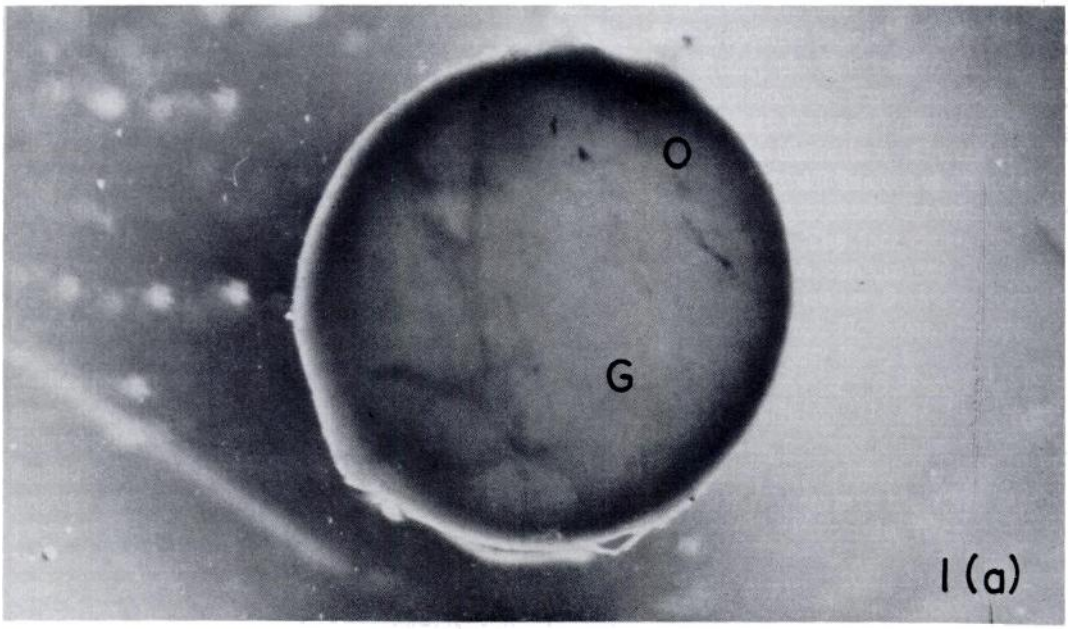
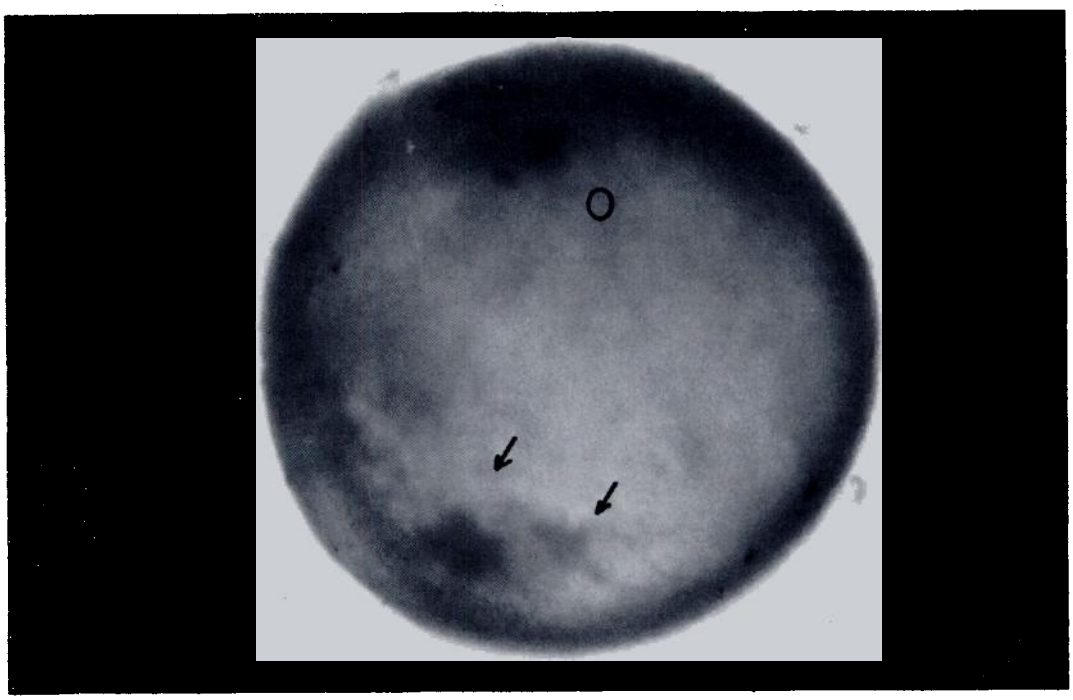
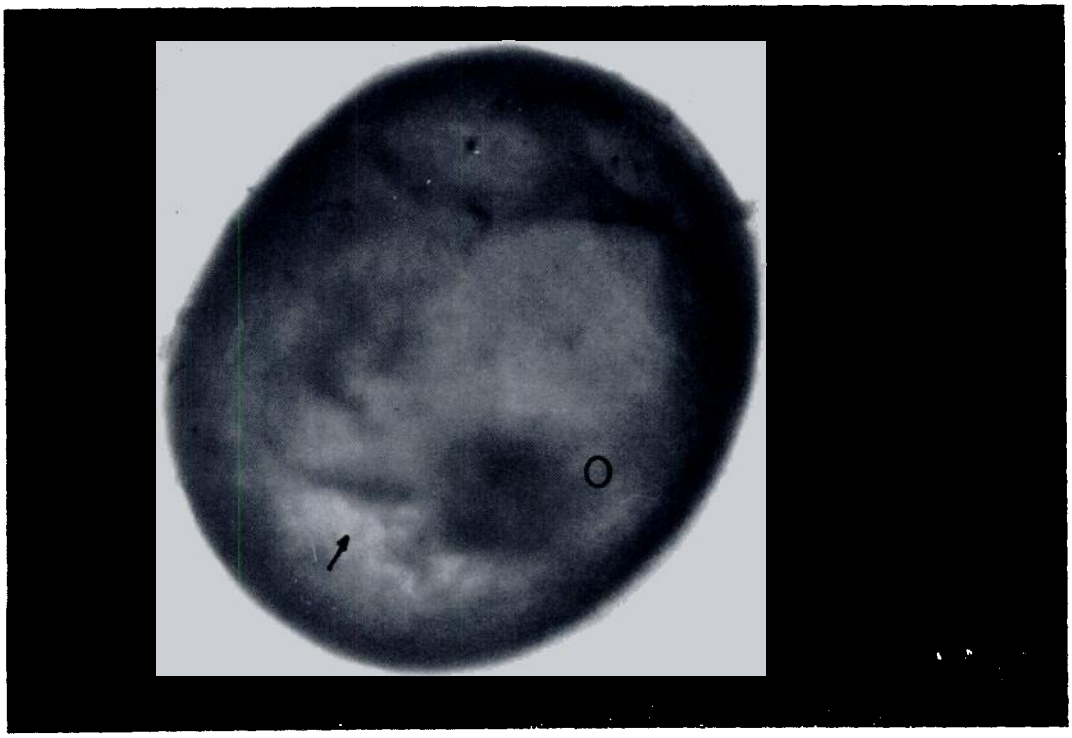
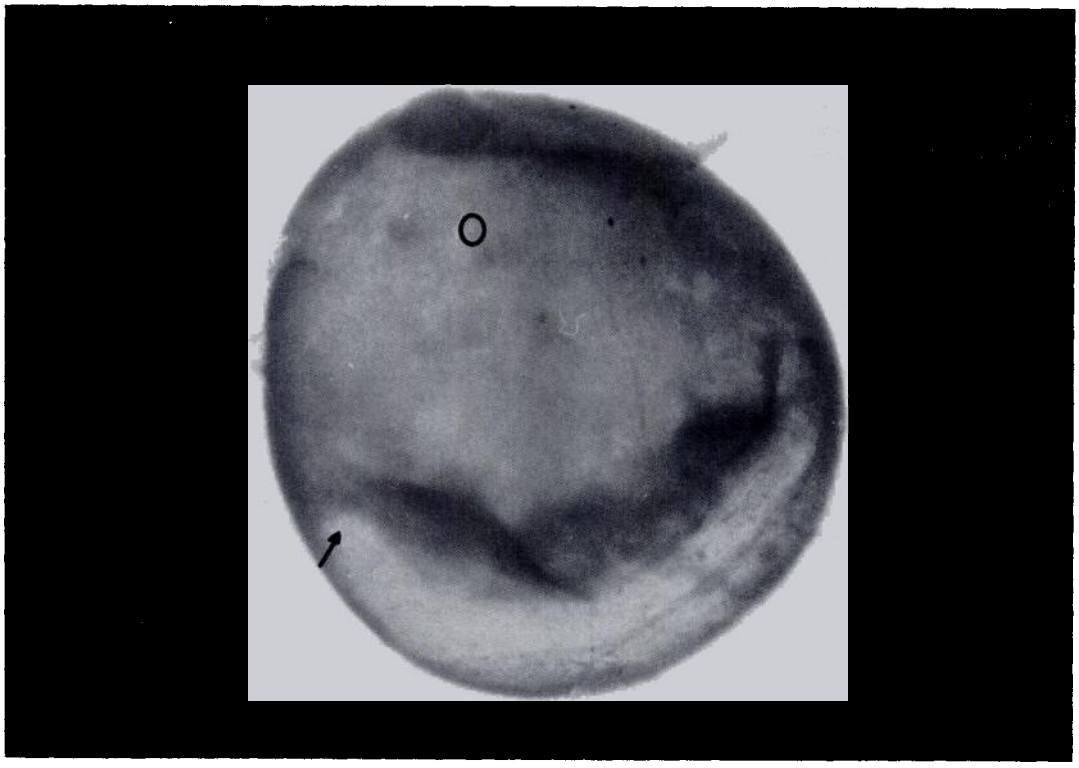
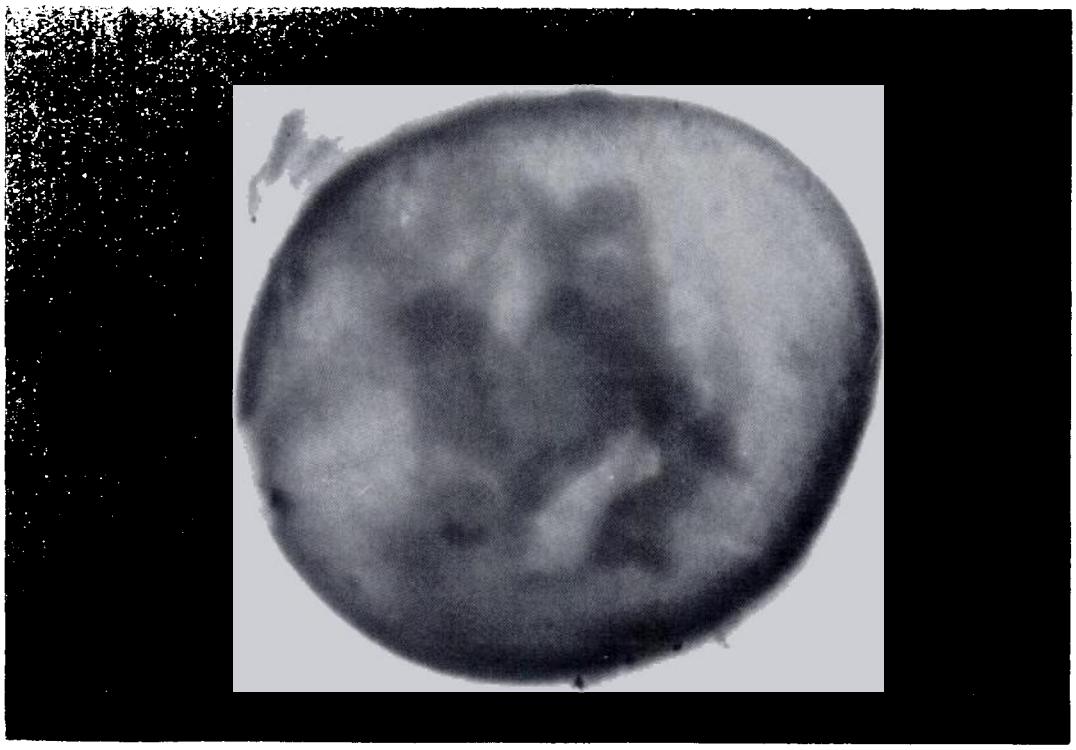


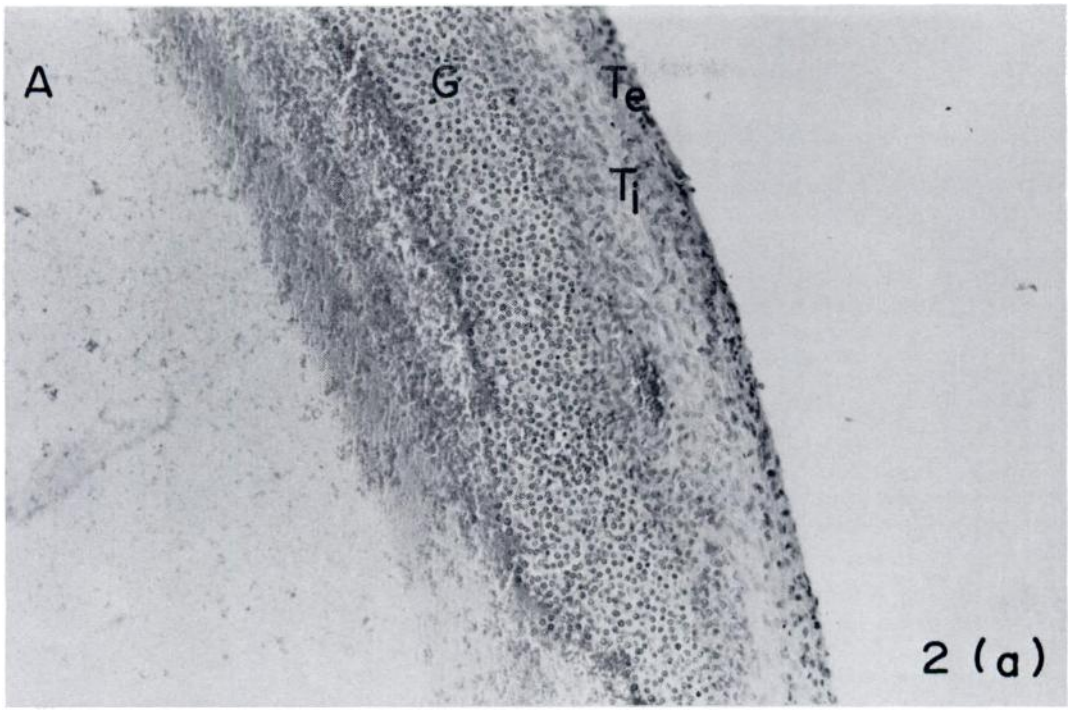
FIG. 1. Morphological correlates of follicular atresia. Photomicrographs of isolated whole ovine follicles were prepared using transmitted light. a) Stage 1 atresia; a continuous membrana granulosa (G), oocyte-cumulus complex (O) and thecal capillaries are visible. X 30. b) Stage II atresia; the oocyte-cumulus complex remains obvious and membrana granulosa exhibits some discontinuity (→). Thecal capillaries are generally visible. X 15. c) Stage III atresia; thecal capillaries and the oocyte-cumulus complex are less evident and distinct perforations of the membrana granulosa (→) are seen. X 20. d) Stage IV atresia; thecal capillaries are absent and the oocyte which is partially denuded of cumulus cells has become detached from the follicle wall as evidenced by its free movement within the follicle. Extensive perforation of the membrana granulosa and loss of contact with the theca has occurred (→). X 11. e) Stage V atresia thecal capillaries and oocyte are absent. Complete fragmentation of the membrana granulosa and detachment from the theca is seen. X 15.











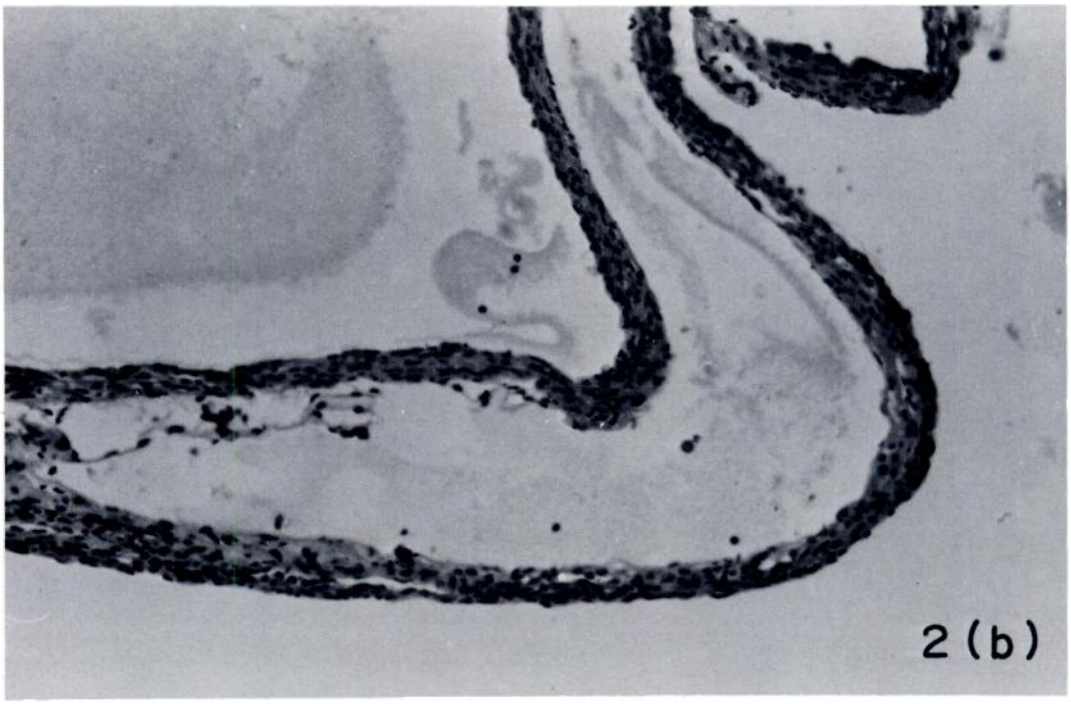


FIG. 2. a) Histology of isolated follicles. Paraffin sections (5 μm) of whole ovine follicles were prepared as previously described (see Materials and Methods). Distinct theca externa (Te), theca interna (Ti), membrana granulosa (G) and follicular antrum (A) are indicated. × 145. b) Histology of an isolated theca fraction. After removal of granulosa cells, paraffin sections of the isolated "theca" fraction were prepared as previously described (see Materials and Methods). Minimal contamination with adherent granulosa cells is evident.

unlabeled gonadotropin from total counts bound.

At the end of the incubation period (27–36 h), each group of follicles was rinsed 3 times in ice cold saline. Individual follicles were hemisected and centrifuged at 250 × g for 15 min at 4°C. Following aspiration of cell-free follicular fluid, the pellet comprising granulosa and theca cells was then resuspended in 1.0 ml ice cold saline by repeated withdrawal into a 1.0 ml disposable plastic tuberculin syringe. The hemispheres of theca were allowed to settle out of the suspension under gravity and the granulosa enriched supernatant removed with a Pasteur pipette. This process was repeated and the resultant pooled granulosa and theca-rich fractions were washed twice by resuspension in 2.0 ml of ice cold saline and centrifuged at 5000 × g at 4°C for 15 min.

Determination of γ-activity (Packard Autogamma) and protein content (Lowry et al., 1951) of the tissue pellet enabled results to be expressed as counts specifically bound per mg of protein (cpm/mg).

Student's t test and one-way analysis of variance were used in the statistical evaluation of data.

RESULTS

Dissection of Follicles and Separation of Granulosa from Theca Cells

Follicles dissected from ovine ovaries were free of stromal tissue and exhibited a normal theca and membrana granulosa (Fig. 2a).

Separation of theca from granulosa cells at the end of incubation gave 2 distinct fractions. When examined histologically, sheets of theca were seen to be relatively free of granulosa cells (Fig. 2b). Also, no thecal elements were evident in the granulosa fractions.

Histological Confirmation of the Morphological Correlates of Atresia

When examined histologically, the degree of integrity of the membrana granulosa was

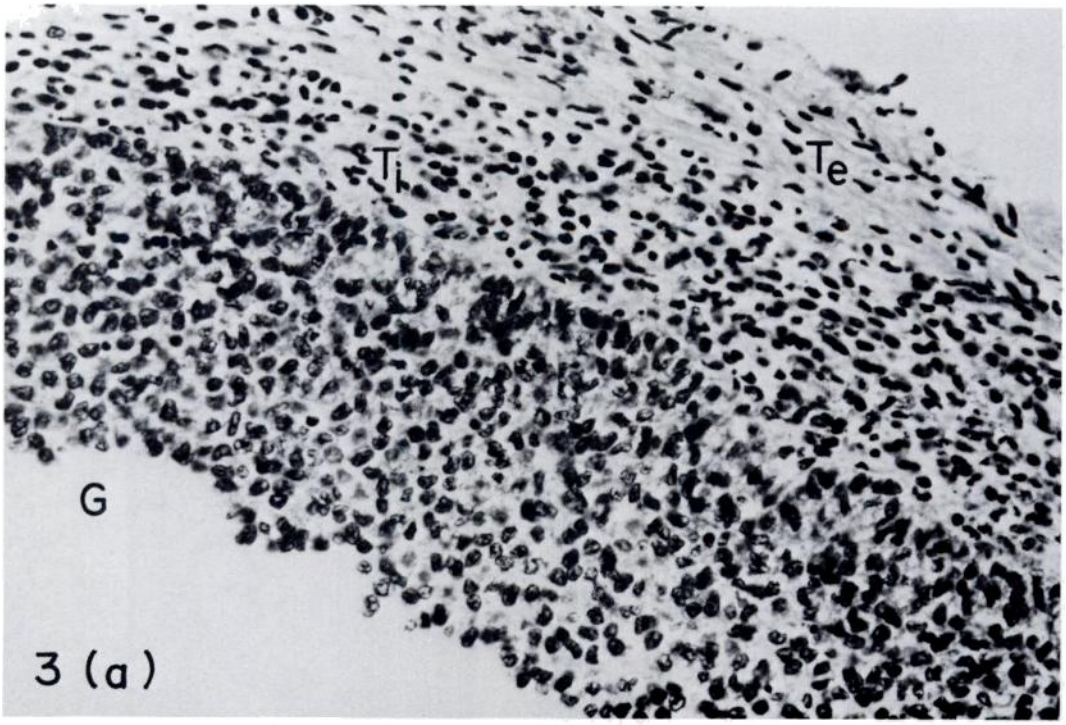
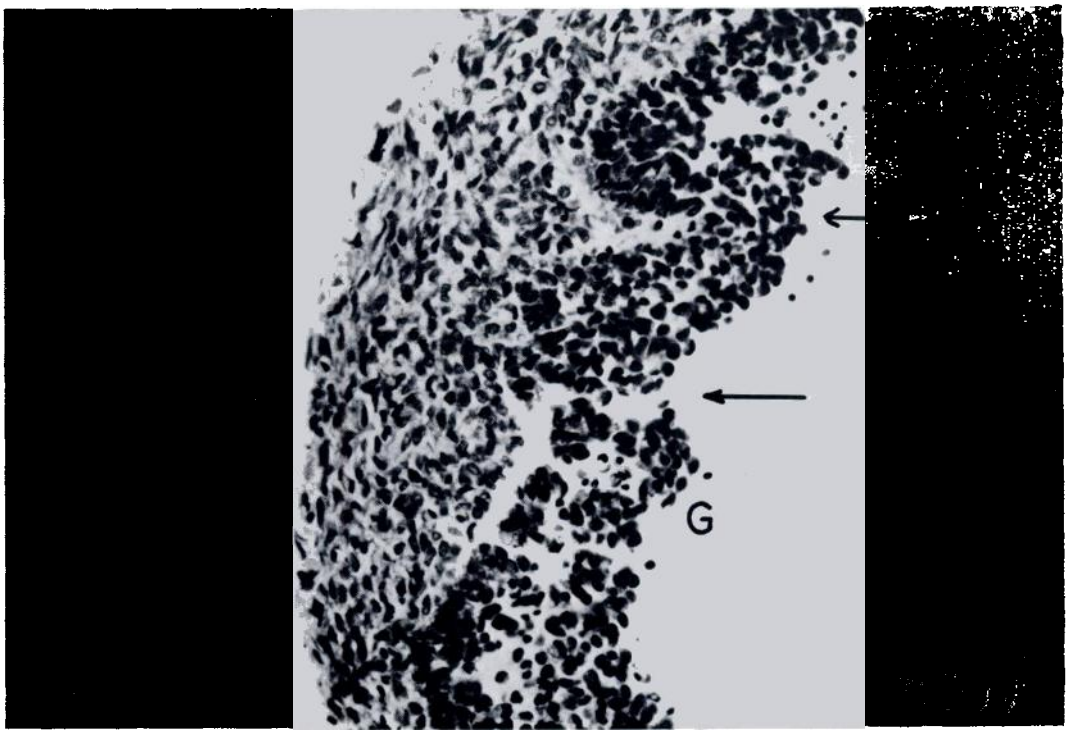
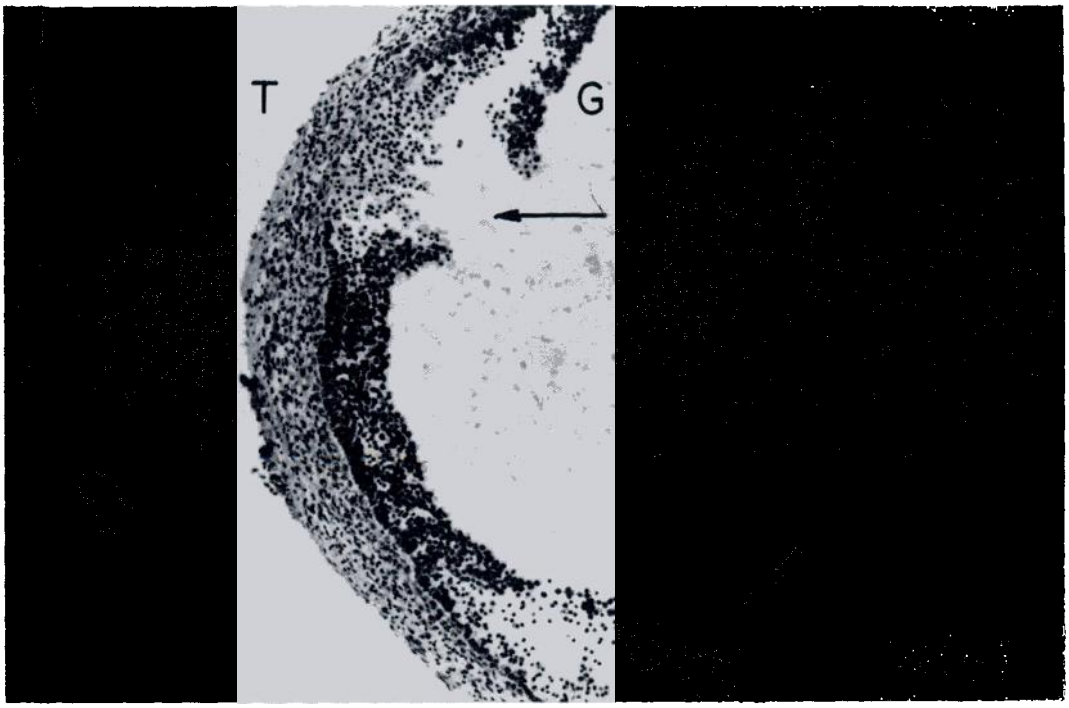
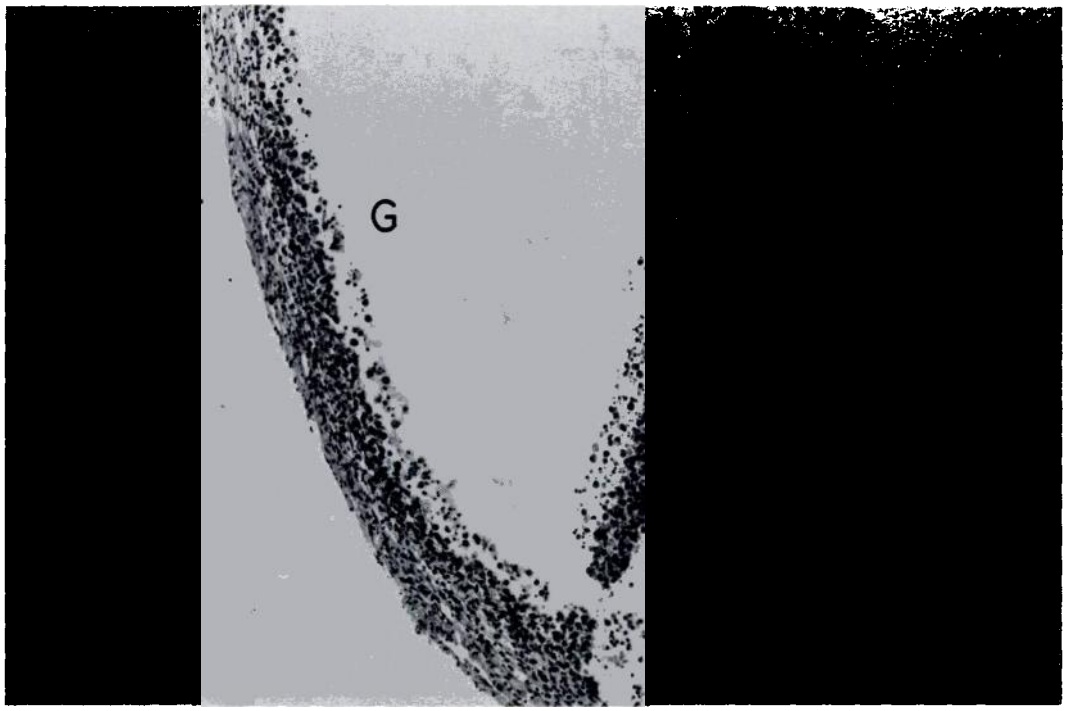
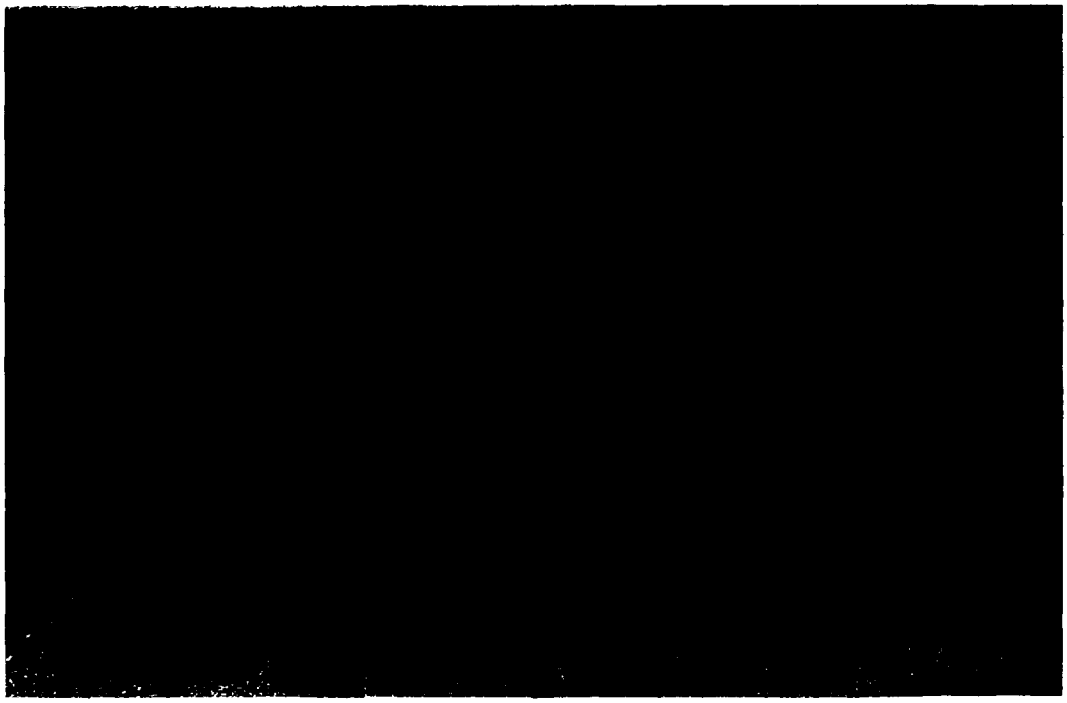


FIG. 3. Histological confirmation of the chosen morphological correlates of follicular atresia. Isolated ovine follicles were assigned to one of five stages of morphological atresia prior to histological examination (see Materials and Methods). a) Class I atresia; theca interna (Ti) and theca externa (Te) are evident and in close contact with the uniformly arranged cells of the membrana granulosa (G). Very few pyknotic nuclei are present. × 450. b) Class II atresia; localized areas of folded granulosa with some discontinuity (→) and pyknosis of granulosa cells. × 360. c) Class III atresia; local perforations of the membrana granulosa are associated with areas of extensive pyknosis of granulosa cell nuclei (→). × 144. d) Class IV atresia; perforation of the membrana granulosa is extensive and partial separation from the theca interna has occurred. Pyknotic nuclei are evident in both theca and granulosa cells. × 144. e) Class V atresia; the residual fragments of the membrane granulosa are entirely separated from the theca. × 144.









related directly to the stage of morphological atresia as judged using transmitted light (Figs. 3a-e). Integrity of the membrana granulosa decreased as the degree of morphological atresia increased. Follicles of Class I atresia exhibited a unifrom membrana granulosa in close contact with the theca interna (Fig. 3a), while the membrana granulosa of Class V follicles was fragmented and separated from the theca interna (Fig. 3e). In all cases, regions of discontinuity of the membrana granulosa were associated with pyknosis of granulosa cell nuclei.

Incidence of Atresia as a Function of Follicular Diameter

Examination of 4415 isolated follicles indicated that the incidence of atresia was dependent upon follicular diameter (Table 1). When analyzed by means of a 5 × 5 test of independence, this relationship was statistically significant ($P<0.01$).

Of those follicles less than 1 mm diameter, 147 of the 174 (79%) were essentially nonatretic (Classes I and II), whereas 54 of the 58 (93%) follicles of greater than 6 mm diameter were atretic (Classes III, IV and V) (Table 1).

Equilibration of [^{125}I]-labeled Gonadotropins between Incubation Medium and Follicular Fluid

Transfer of [^{125}I]-hFSH and [^{125}I]-hCG from incubation medium to follicular fluid was time dependent. Equilibrium was reached at 20 and 25 h for [^{125}I]-hFSH and [^{125}I]-hCG, respectively, with a partition coefficient of 0.76 being reached at equilibrium. These observations were unaffected by either an excess of unlabeled gonadotropin (200 IU/ml) or by stage of atresia (Figs. 4a, b).

Binding of [^{125}I]-Labeled Gonadotropins to Theca and Granulosa Cells as a Function of Follicular Diameter

Specific binding [^{125}I]-hFSH to granulosa cells decreased from 38.1 ± 1.2 cpm × 10^3/mg for follicles of less than 2 mm diameter to 22.3 cpm × 10^3/mg for follicles of >6 mm diameter ($P<0.05$). Mean specific binding of [^{125}I]-hFSH to theca cells was seen to increase slightly with increased follicular diameter although the extent of binding was limited in comparison to that of the granulosa (Fig. 5a).

Specific binding of [^{125}I]-hCG to granulosa cells increased from 2.4 ± 0.4 (<2 mm diameter) to 5.2 ± 4.1 (2-4 mm diameter) and 51.0 ± 0.9 cpm × 10^3/mg (4–6 mm diameter). Binding to the granulosa of follicles 4–6 mm diameter was significantly greater than to that of follicles less than 4 mm diameter ($P<0.0025$). Theca cells from follicles 4–6 mm diameter bound significantly less [^{125}I]-hCG (30.2 ± 7.8 cpm × 10^3/mg) than theca cells from follicles of 2–4 mm diameter (42.1 ± 2.5 cpm × 10^3/mg) and less than 2 mm diameter (42.2 ± 8.9 cpm × 10^3/mg) ($P<0.05$; Fig. 5b).

Nonspecific binding of both [^{125}I]-hFSH and [^{125}I]-hCG ranged from 10–18% of total counts bound.

Binding of [^{125}I]-labeled Gonadotropins to Granulosa Cells as a Function of Atresia

A decrease in mean specific binding of [^{125}I]-hFSH and [^{125}I]-hCG to granulosa cells was associated with increasing degrees of atresia within any size group (Figs. 6a, b).

Upon analysis of variance, the amount of [^{125}I]-hFSH bound to granulosa cells varied as a function of follicular atresia rather than follicular diameter ($P<0.05$). Conversely, while the amount of [^{125}I]-hCG bound to granulosa

TABLE 1. Incidence of atresia as a function of follicular diameter. Distribution of 4415 isolated follicles with respect to follicular diameter and stage of morphological atresia.

Stage of morphological atresia	Follicular diameter (mm)				
	<1	1–2	2–4	4–6	>6
I	78	225	54	0	0
II	59	644	369	26	4
III	28	743	719	71	19
IV	5	416	458	71	23
V	4	139	214	34	12
Total number of follicles within size group	174	2167	1814	202	58

cells was decreased with increased atresia, the overall extent of binding was determined by follicular diameter ($P<0.01$).

When analyzed independently of follicular diameter, the mean specific binding of [^{125}I]-hFSH to granulosa cells of Class V follicles (5.9 ± 3.1 cpm/mg) was significantly less than that of all other classes of atresia (11.3 ± 5.4 to 22.8 ± 4.0 cpm/mg) ($P<0.05$).

The mean specific binding of [^{125}I]-hCG to granulosa cells of stage IV and V follicles in combination was significantly less than that to follicles of stage I or stage II atresia for all size ranges examined ($P<0.05$).

DISCUSSION

Data obtained in this study are representative of gonadotropin binding to follicular elements, as stromal tissue was completely eliminated by the dissection of follicles from surrounding

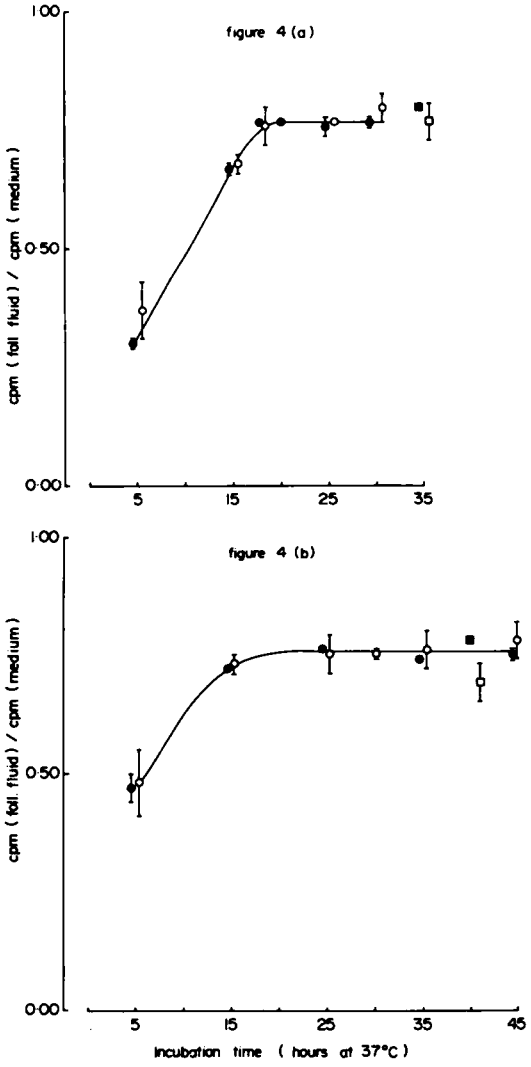


FIG. 4. Equilibration of a) [^{125}I]-hFSH and b) [^{125}I]-hCG between incubation medium and follicular fluid of nonatretic (Classes I, II) and atretic (Classes III, IV, V) follicles *in vitro* at 37°C. Each point represents the mean (±SD) of duplicate determinations (n = 8–14). Classes I, II (●); III–IV (○), Classes I, II plus excess unlabeled gonadotropin (200 IU/ml) (■); III–V plus excess unlabeled gonadotropin (200 IU/ml) (□).

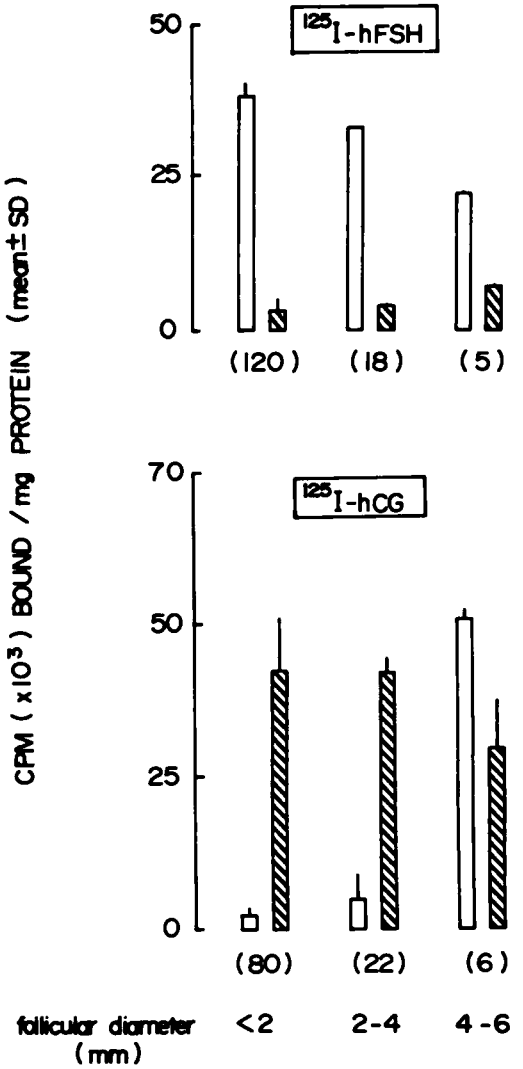


FIG. 5. Specific binding of a, top) [^{125}I]-hFSH and b, bottom) [^{125}I]-hCG to granulosa □ and theca cells ■ of whole ovine follicles as a function of follicular diameter. Numbers in parentheses denote numbers of follicles.

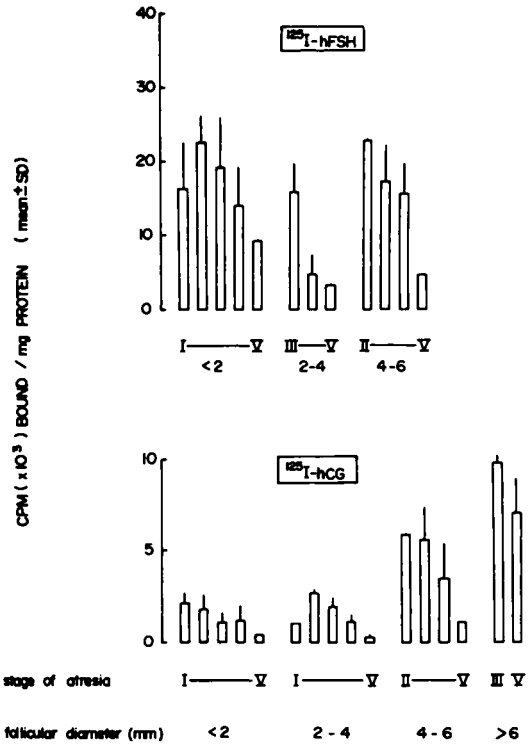


FIG. 6. Specific binding of a, top) [^{125}I]-hFSH and b, bottom) [^{125}I]-hCG to granulosa cells as a function of follicular atresia.

ovarian tissue. Similarly, isolated granulosa cells were entirely free of thecal elements, although some contamination of the thecal fraction by adherent granulosa cells was possible.

Changes in morphology of the ovine follicle have recently been associated with follicular atresia (Hay et al., 1976; Turnbull et al., 1977; Moor et al., 1978; O'Shea et al., 1978). In this study, histological confirmation of similar morphological correlates further supports the use of such morphological features as a basis for assessment of follicular atresia. Discontinuities of the membrana granulosa were invariably coincident with increased pyknosis of granulosa cell nuclei, indicating that these morphological changes were not a consequence of mechanical damage sustained during dissection of follicles from the ovarian stroma.

Macroscopic assessment of atresia has in this instance proven useful. Howver, the morphological changes associated with atresia are surely secondary to other more central biochemical events responsible for the onset of atresia. Although histological confirmation of the changes in follicular morphology associated with atresia was possible, no biochemical confirmation has as yet been undertaken.

The distribution of isolated follicles with respect to both stage of atresia and follicular diameter suggests that the incidence of atresia in sheep ovarian follicles is directly related to follicular diameter and that a follicle may undergo atresia at any stage of growth, although large follicles (>4 mm) are more likely to be atretic than small follicles (<4 mm). Since not all follicles were dissected from each ovary a comparison of the total number of follicles recovered in each size group or stage of atresia is not justified. However, the total number of follicles recovered in each size group reflects the probability of a follicle being found in that state and thus the time that a follicle spends in such a state. Consequently, the time required for an increase in follicular diameter from 1 to 2 mm is greater than that required for any of the subsequent increments of 2 mm. This finding corroborates the previous study of Turnbull et al. (1977) in which 65% of follicles examined were of 1.2–5 mm diameter and the same inference drawn. As indicated by mitotic indices, the maximal growth rate of granulosa cells occurs in follicles of 1–2.5 mm diameter (Turnbull et al., 1977). This implies that the more rapid increases in follicular diameter of large follicles is due to increased antral volume rather than proliferation of granulosa cells.

An inability of excess unlabeled gonadotropin to alter the observed equilibration kinetics suggests that movement of [^{125}I]-labeled gonadotropin into whole follicles *in vitro* is by simple diffusion. As such, the differences in molecular weight that exist between hFSH (MW 33,000) and hCG (MW ∿50,000) account for the differences in time required for complete equilibration of each [^{125}I]-labeled gonadotropin between incubation medium and follicular fluid. Only 76% of either labeled gonadotropin was able to be assimilated into follicular fluid. In view of the proposed mechanism of transfer between incubation medium and follicular fluid, the [^{125}I]-labeled gonadotropin remaining in the incubation medium was of a higher molecular weight form. Neither the rate at which [^{125}I]-labeled gonadotropin entered follicles nor the ultimate equilibrium attained was different when atretic and non-atretic follicles were compared. The decreased binding of [^{125}I]-labeled gonadotropin to atretic follicles cannot therefore be attributed

to differences in equilibration of labeled gonadotropin between incubation medium and follicular fluid.

These findings serve merely to define the *in vitro* system as used and in no way suggest that similar equilibration times exist *in vivo*, where gonadotropins are delivered to the follicle via intrathecal capillaries and thus do not have to traverse the entire thecal structure.

Maximal binding of [^{125}I]-labeled gonadotropins to suspensions of granulosa cells from other species requires 3–6 h *in vitro* (Nakano et al., 1977; Nimrod et al., 1976). Thus, the time course of binding of [^{125}I]-labeled gonadotropins to granulosa cells in this study would be expected to parallel the equilibration curve, with a displacement of 3–6 h. Consequently, the incubation times chosen in this study should have enabled maximal binding of [^{125}I]-labeled gonadotropin to granulosa cells of whole follicles *in vitro*.

The data indicate that changes in the binding of [^{125}I]-hFSH and [^{125}I]-hCG to whole follicles *in vitro* are associated with follicular maturation in the ewe and that these changes are attributed to alterations in the capacity of the granulosa rather than theca cells to bind [^{125}I]-labeled gonadotropin. In addition, while the granulosa is able to bind both [^{125}I-labeled gonadotropins, binding of [^{125}I]-hFSH to the theca was limited in comparison to the binding of [^{125}I]-hFSH to granulosa cells and could in part be due to contamination of the thecal tissue with adherent granulosa cells. This confirms that the site of action of hCG in the ovary is the granulosa and theca cells and that of FSH the granulosa cell alone.

Nonatretic follicles of all sizes examined had a similar capacity to bind [^{125}I]-hFSH, while the binding of [^{125}I]-hCG was greatly increased in large follicles. This suggests that nonatretic follicles of all sizes examined are responsive to FSH and that maximal sensitivity to hCG (LH) is attained only in large preovulatory follicles. The relationship of the decreased binding capacity of granulosa cells for each of the [^{125}I]-labeled gonadotropins which is associated with morphological atresia is most probably a consequence rather than a cause of the atretic process, as distinct morphological signs of atresia are evident prior to an significant change in binding capacity. Thus, the binding of [^{125}I]-hFSH and [^{125}I]-hCG to follicles *in vitro* is significantly correlated with the stage of morphological atresia and follicular diameter, respectively. Previous studies (Nakano et al., 1977; Channing and Kammerman, 1973) have associated changes in follicular binding capacities with changes in follicular diameter without due regard for the degree of follicular atresia. Such changes are probably a consequence of the combined effects of decreased binding of [^{125}I]-labeled gonadotropins to atretic follicles and the changes in the incidence of atresia that exist between groups of follicles of different follicular diameter.

It may be argued that binding of [^{125}I]-labeled gonadotropins to follicles *in vitro* is not a true measure of follicular responsiveness to gonadotropins. To substantiate these findings, further studies are presently being undertaken to evaluate changes in biological responses to gonadotropin during follicular growth and atresia.

In conclusion, changes in the capacity of the follicular granulosa cells to bind [^{125}I]-hFSH and [^{125}I]-hCG are associated with follicular atresia and growth. It is necessary that the degree of atresia be considered in addition to follicular diameter when interpreting follicular gonadotropin binding data.

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