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An Enzymatic Method for Dissociation of Intact Follicles from the Hamster Ovary: Histological and Quantitative Aspects

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ABSTRACT

An enzymatic method was developed to collect intact follicles at different stages of development from cyclic hamsters to study ovarian folliculogenesis under various circumstances. Ovaries from 6 adult hamsters on each day of the cycle (Day 1=ovulation) were collected, corpora lutea and large preantral and antral follicles were dissected, and follicles saved. Minced ovaries were then incubated with a mixture of collagenase, DNAse and pronase at 37°C for 20 min to disperse intact follicles. Histological studies with 2191 isolated follicles revealed 10 different stages of follicular development (depending on the number of granulosa cell layers surrounding the oocyte and development of the antrum). Of the total follicular population, 14% showed signs of atresia, with 50% of those having 1–3 layers of granulosa cells (Stages 1–3); a second peak of 18% was observed in antral follicles (Stages 8–10). No signs of thecal cells were evident until the follicles reached Stage 6 (7–8 layers of granulosa cells), which possibly accounts for reduced atresia in this class and beyond. Ultrastructural study revealed that there were no signs of morphological damage to the basement membrane or to other subcellular organelles in the small preantral follicles. The presence of subnuclear lipid droplets in follicles with 3 layers of granulosa cells provided evidence for potential steroidogenesis by small follicles. The number of Stage 1–10 follicles was remarkably constant throughout the estrous cycle (460 ± 34 per animal on Day 1 vs. 492 ± 66 on Day 4). The usefulness of this method in analyzing follicular kinetics is illustrated in experiments involving hypophysectomy and the effects of unilateral ovariectomy. This procedure offers an improved method to study the factors responsible for the growth and the differentiation of small preantral follicles in the mammalian ovary.

INTRODUCTION

Although considerable attention has been paid to the hormonal control and function of large ovarian follicles, the contribution of smaller follicles to ovarian activity is still an open question. The mammalian ovary possesses a large number of follicles ranging from primordial to preovulatory stages (for references see Pedersen and Peters, 1968; Greenwald, 1974). Isolation of antral and preantral follicles by microdissection has enhanced our understanding of the direct interaction of hormones and other factors on large follicles (e.g., Mills and Savard, 1972; Bogovich and Richards, 1982; Terranova and Garza, 1983). However, such studies have not been extended to smaller follicles because of the lack of a suitable method of separation. A technique for isolating follicles from rat and mouse ovaries utilizing enzymatic digestion with pronase was devised by Grob, who subsequently studied the steroidogenic potential of the follicles in vivo (Grob, 1964) and in vitro after monolayer culture (Grob, 1971). A similar study was carried out by Nekola and Nalbandov (1971). However, treatment with proteolytic enzymes—pronase for example—can be detrimental to cell membranes (Kono, 1969). A more gentle procedure for isolating rabbit ovarian follicles, using collagenase, was described by Nicosia and co-workers (1975). Their study clearly demonstrated morphological and functional normality of the isolated follicles.

Ovarian follicles have been classified into 7 stages in the hamster according to their size and number of surrounding layers of granulosa cells (Moore and Greenwald, 1974). The hormonal regulation of steroidogenesis in large antral follicles in the hamster is well documented (see Greenwald, 1978) but virtually nothing is

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known about the role of hormones and other factors in the development of primary and small secondary follicles. The present study was therefore directed towards developing a simple and rapid technique for recovering large numbers of morphologically and functionally intact follicles at different stages of folliculogenesis and to gather some fundamental information about the follicular population.

MATERIALS AND METHODS

Preparation of the Dissociating Medium

The principle is based on the loosening of the collagenous matrix by enzymatic digestion and dispersion of small preantral follicles by gentle mechanical agitation. Shortly before incubation of the ovaries, collagenase from *Clostridium histolyticum* (Sigma Type I, 482 units/mg solid, Sigma, St. Louis, MO) was dissolved in Medium 199 with 25 mM Hepes (Gibco, Grand Island, NY) at a pH of 7.0 and a concentration of 5 mg per 1.5 ml. A 500-μl aliquot of a DNAse solution (DNAse, bovine pancreas III, 1350K units/mg solid; 400 units/ml, Sigma) and 50 μl pronase (*Streptomycis greiseus*; pronase XIV, 5.8 units/mg solid, 100 mg/100 ml; Sigma) dissolved in Medium 199 were added and the mixture was prewarmed to 37°C (total volume=2 ml).

Preparation of Tissue

Adult, virgin golden hamsters (*Mesocricetus auratus*) with at least 3 regular 4-day cycles were used, with the cycle monitored by the postovulatory vaginal exudate (Day 1 of cycle; Day 4-proestrus). For each day of the cycle, 6 animals were decapitated between 0900–1000 h, the ovaries quickly removed and placed in ice-cold Medium 199, and fat and connective tissue were removed. Large preantral and antral follicles (Stages 6–10; defined in Table 1) and corpora lutea were dissected from the ovaries and the follicles cleaned and saved. The ovaries were then minced and incubated in the prewarmed enzyme solution for 20 min in a Dubnoff metabolic shaker at a temperature of 37°C with rapid shaking. Subsequently, 1% bovine serum albumin in Medium 199 was added, and the mixture centrifuged at 400 rpm at 4°C for 3 min. The supernatant was discarded, the pellet resuspended in fresh Medium 199 and dispersion of follicles completed by gentle mechanical agitation with a Pasteur pipette. The suspension was then filtered through nylon mesh (350 μm) and centrifuged, the pellet resuspended in Medium 199 containing 5 mM EGTA (Sigma), and follicles of different sizes were harvested.

In the latter part of the study, the method was improved by including additional mechanical agitation with a Pasteur pipette at the end of the first 10 min of incubation. This modification led to a severalfold increase in the yield of follicles so that in the final experiments instead of pooling the ovaries of 6 animals, collection of follicles from 1 animal was feasible. The entire procedure for three pairs of ovaries took approximately 4 to 5 h.

After incubation with the enzymes, the follicles were sorted into different diameter ranges with the aid of precalibrated micropipettes, the number of follicles recorded, the follicles fixed in Bouin's fluid for 3 h before being placed in agar blocks for histological processing. Sections were cut at 7 μm and stained with hematoxylin and eosin.

The method of classification was adopted from Moore and Greenwald (1974) with minor modifications. Follicles were placed in 10 stages; the first 6 were based on the layers of granulosa cells surrounding the oocyte, the 7th stage corresponded to follicles with beginning of antral cavity formation, and Stages 8–10 included Graafian follicles of increasing sizes. Follicles were studied for the number of layers of granulosa cells in relation to diameter, onset of thecal cell association, and signs of atresia.

A group of follicles with 2 and 3 layers of granulosa cells, dispersed enzymatically from the ovaries, were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h, postfixed in 2% osmium tetroxide, dehydrated in grades of ethanol and propylene oxide and finally embedded in Epon. Ultrathin sections (0.1 μm) were cut with an LKB ultratome, stained with uranyl nitrate and lead citrate and examined on a JOEL electron microscope. This was done to establish any possible structural damage to the follicle attributable to the entire procedure and to determine whether any thecal precursor cells were present.

Statistical Analysis of Data

Numbers of follicles at different stages obtained at different times on each day of the estrous cycle and after various experimental manipulations were analyzed statistically by analysis of variance (ANOVA) with the help of the BMDP program and Duncan's multiple range test. Values were considered statistically significant when $P<0.05$.

RESULTS

The technique used for enzymatic dissociation of the ovary yielded large numbers of intact follicles from primary to large preantral Stages 1 to 5 (Figs. 1–11). Stages above this size were dissected by hand. Moreover, the total number of follicles recovered was satisfactory using the first method in which ovaries from 6 animals were pooled for each day of the cycle. The results were excellent with the improved method involving repeated agitation during the first 10 min of incubation, which consequently freed follicles otherwise trapped in large tissue fragments. In fact, with the latter modifications, sufficient small preantral follicles were harvested from one pair of ovaries to make the technique of value in innumerable situations such as in vitro incorporation of [^{3}H]thymidine, steroidogenic response to gonadotropins and other substances, and changes in peptide hormone receptor populations.

From the experiment in which ovaries were pooled from 6 animals on each day of the cycle, 2191 follicles were sectioned serially and

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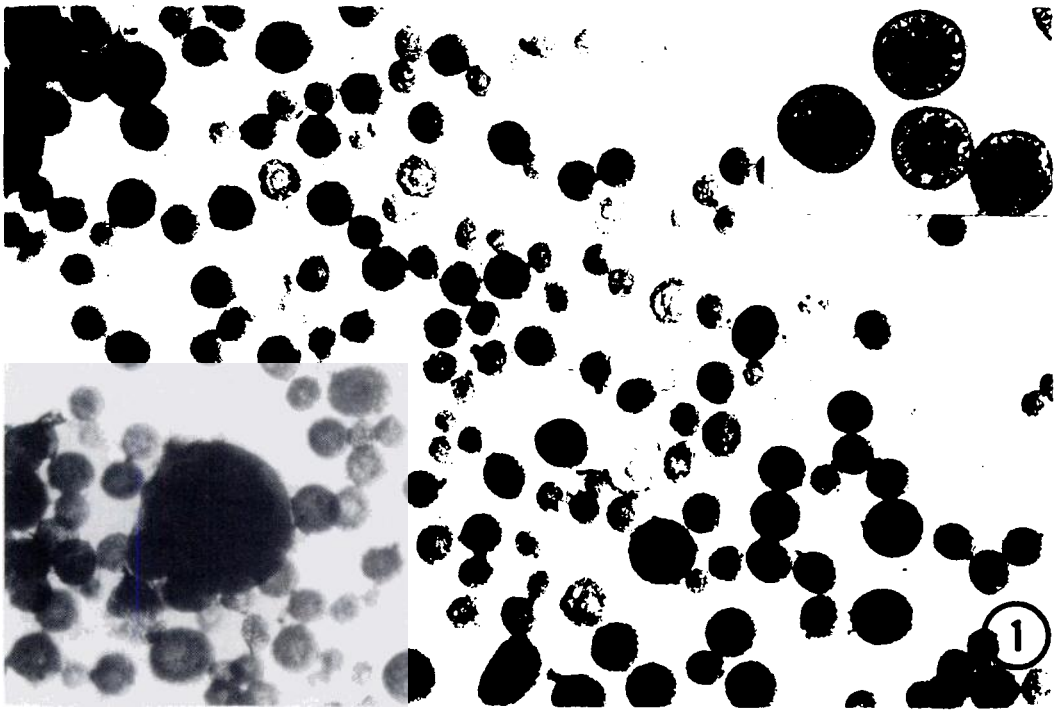

FIG. 1. Photomicrograph of hamster ovarian follicles showing different size ranges isolated by enzymatic dissociation.Smaller follicles represent large Stage 1 and Stage 2 follicles. Smaller Stage 1 follicles cannot be distinguished at low magnification. X69. *Insert* shows magnified view of small follicles. X133.

classified according to size and whether they were atretic or normal. Histological criteria for atresia were nuclear pyknosis, leukocyte infiltration and degeneration of the oocyte. Approximately 14% of the total population of follicles was atretic, with 50% of the total number of atretic follicles in Stages 1–3 with 1–3 layers of granulosa cells (Table 1). There was a sharp drop in the number of atretic follicles once they were surrounded by 7 or 8 layers of granulosa cells, followed by a second peak of atresia in antral follicles (Table 1).

It was interesting that follicles were not surrounded by a thecal layer until there were 7 or 8 layers of granulosa cells (Fig. 8). To check on whether the enzymatic treatment was responsible for the removal of the thecal shell, Stages 1–5 follicles were separated by mechanical agitation by repeated aspirations with a Pasteur pipette. Under these circumstances, the small secondary follicles still lacked a thecal component.

The ultrastructure of isolated small follicles confirmed the feasibility of this approach for experimental studies. There were no signs of damage to basement membranes and the mitochondria and other organelles were well maintained (Fig. 12). For the granulosa cells, the nucleus-to-cytoplasm ratio was large and the cells were well connected by desmosomes. In the Stage 3 follicle, the peripheral layer of granulosa cells had abundant subnuclear lipid droplets while cells lying closest to the oocyte had few or no droplets (Fig. 12). The oocytes were also well preserved, providing additional proof of the morphological normality of the follicles collected by enzymatic dissociation of the ovary (Fig. 13).

Table 2 shows the relationship between diameter of the follicles correlated with the stage of follicular development. The range in diameter is based on the use of precalibrated pipettes which were used to collect follicles of different sizes. There was a fairly wide range in diameter for each stage; e.g., follicles with one layer of granulosa cells (Stage 1) varied from 28 to 74 μm in diameter. However, for each stage, the majority of follicles fell within a circumscribed range (indicated by the *underlined diameter*, Table 2). Based on these subjective

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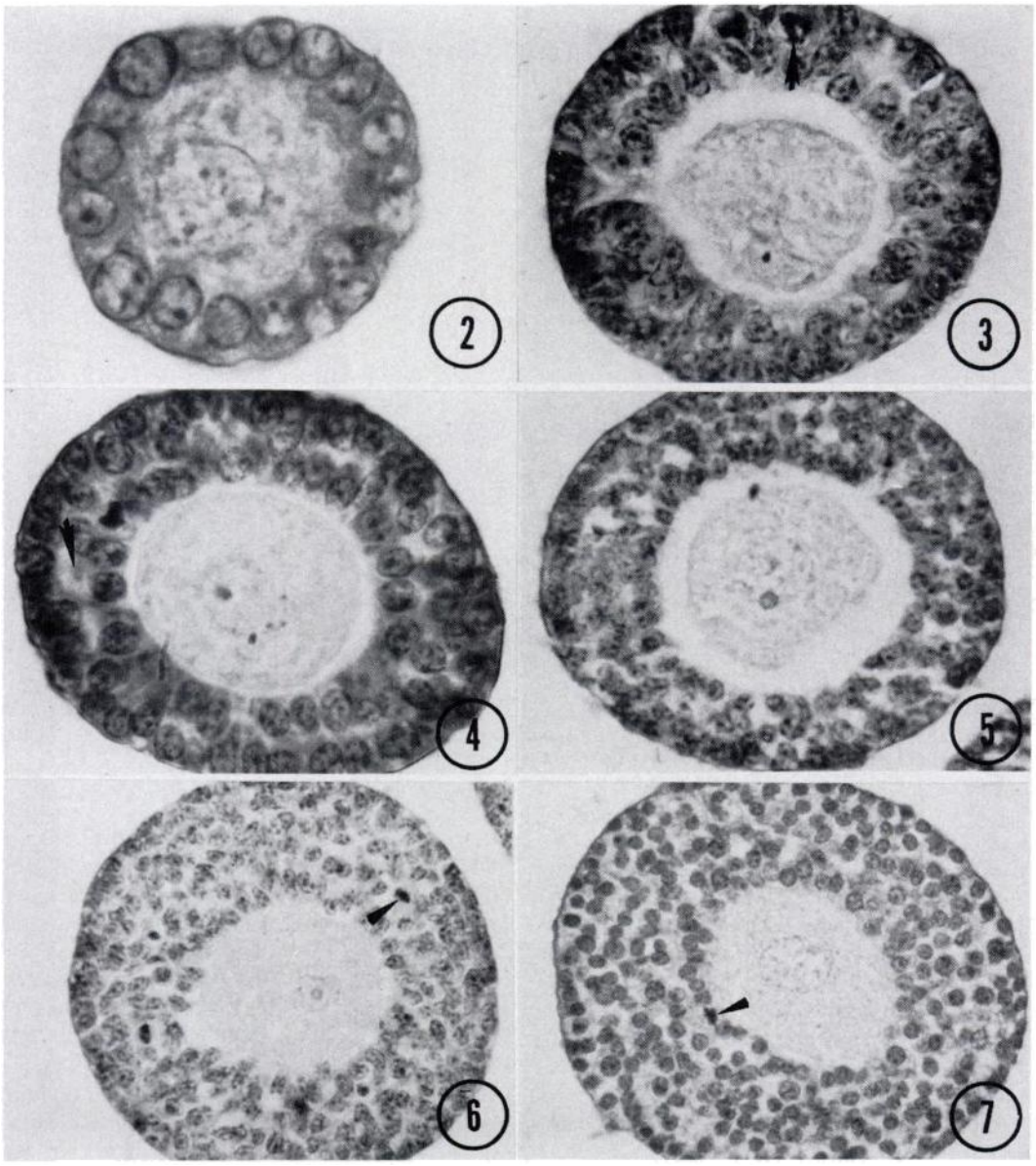

FIG. 2–7. Cross-sections of healthy follicles dissected enzymatically from the hamster ovary.

FIG. 2. A primary follicle with a single layer of granulosa cells (Stage 1). Note that the basement membrane is intact and all granulosa cells have large nuclei with cells intimately attached to the vitelline membrane of the oocyte. No signs of cellular necrosis are evident. ×970.

FIG. 3. A bilaminar follicle with healthy-looking granulosa cells and oocyte (Stage 2). Large nuclear-to-cytoplasm ratio indicates that these cells are in an active growth phase. *Arrow* indicates cells in mitosis. ×677.

FIG. 4. A trilaminar, secondary follicle with large healthy oocyte (Stage 3). Granulosa cells are in the midst of forming the third layer as evident from the incomplete middle layer (*arrow*) in some areas. ×666.

FIG. 5. A quadrilaminar follicle with a healthy oocyte (Stage 4). The space between the oocyte and granulosa cell layer represents shrinkage due to histological processing. There is no sign of atresia. ×497.

FIG. 6. A secondary follicle with five layers of granulosa cells (Stage 5). Absence of nuclear pyknosis indicates the normality of the follicle. Cells in mitosis are clearly visible (*arrowhead*). ×648. Note that none of the follicles in Figs. 2–6 possesses any trace of thecal cell layer.

FIG. 7. A six cell-layered secondary follicle with normal appearance (Stage 5). Prominent thecal cells are still not present. Follicles at this stage are obtained by both microdissection and enzymatic dissociation. *Arrowhead* indicates a cell in mitosis. ×648.

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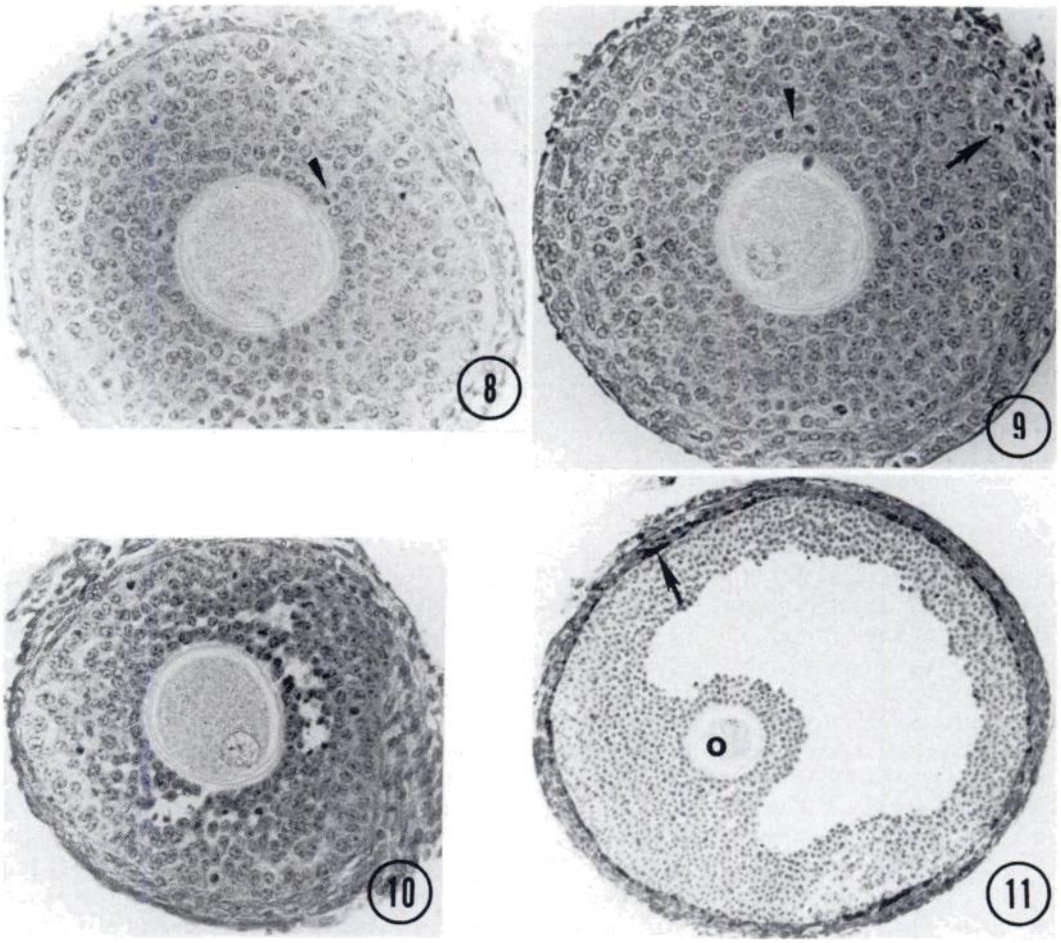

FIG. 8. Cross section of a preantral follicle with seven layers of granulosa cells showing normal cellular morphology (Stage 6). Cells in active mitoses are clearly visible (*arrowhead*). Healthy-looking oocyte is also present. Note the prominent appearance of thecal cell layer which is still not well developed (bilaminar), suggesting the possibility of definitive onset of thecal cell formation between Stages 6 and 7. A few stromal cells are sometimes found attached to the outer thecal membrane. ×40. From this stage and on, follicles were dissected by hand from the ovaries.

FIG. 9. An 8-cell layered preantral follicle with healthy granulosa cells and oocyte (Stage 6). Many mitotic figures are present (*arrowhead*). A well-developed multilayered thecal shell is clearly seen. Mitoses are also present in thecal cells (*arrow*) demonstrating their active development. Some stromal cells are attached to the thecal folliculi. ×40.

FIG. 10. A follicle showing the initiation of antral cavity formation (Stage 7). A multilayered thecal shell is now prominent. Cells in mitosis are still noticeable. ×28.

FIG. 11. A healthy Graafian follicle with large antral cavity and healthy oocyte (o) embedded in cumulus cells (Stage 10). There is a well-developed thecal cell layer with prominent blood sinuses (*arrow*). ×24.

findings, a series of pipettes were made with an interval diameter which would collect the majority of follicles in a particular stage and satisfactorily minimize overlapping with other stages (Table 2).

As previously mentioned, the yield of follicles was considerably enhanced when a tissue suspension was mechanically agitated 10 min after the start of the enzymatic incubation to break up the ovarian fragments. On Day 1 of the cycle, the largest healthy follicles present were small antral stages (Stage 8); the larger follicles were atretic (Table 3). Over the next 2 days there was a gradual increase in the size of antral follicles, with large follicles (Stage 10) first making their appearance on Day 3. The

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TABLE 1. Population of healthy and atretic follicles in cyclic hamster ovaries.

Stage	Layers of granulosa cells	Range in diameter (μm)	Total number follicles examined (%)	
			Healthy	Atretic
1	1	28– 74	290(16)*	6(2)*
2	2	29–110	675(37)	72(19)
3	3	75–148	403(22)	111(30)
4	4	93–225	66(3.6)	67(17.9)
5	5–6	131–370	35(1.9)	25(6.7)
6	7–8	186–370	25(1.4)	3(0.8)
7	Beginning of antrum	186–550	86(4.7)	15(4)
8	Small antral follicle	279–550	36(2)	29(7.7)
9	Intermediate antral follicle	279–550	113(6.2)	21(5.6)
10	Large antral follicle	390–>550	88(4.8)	25(6.7)
			1817	374

*As percentage of total healthy or atretic follicles.

number of follicles from Stages 1–5 were relatively constant throughout the cycle but note the sharp drop in numbers in Stage 6 and beyond. The mean number of follicles per animal also did not vary throughout the estrous cycle (Table 3). Without histological verification the number of healthy and atretic follicles is obviously unknown, but the results in Table 1 provide a rough approximation for each stage of folliculogenesis.

When animals were hypophysectomized on Day 1 at 0900 h, 4 days later (Day 4) the largest follicles consistently present were preantral stages with 7 to 8 layers of granulosa cells. It is noteworthy that the numbers of Stages 5 and 6 follicles were significantly increased in the hypophysectomized animals compared to Day 4 intact controls, and that this finding was still evident 8 days after hypophysectomy (Table 4). The final experimental manipulation consisted of unilateral ovariectomy on Day 3 of the cycle at 0900 h. This led within 6 h to the significant migration of follicles out of the Stage 4 class into the Stage 5 category, which culminated by the morning of Day 4 in a doubling in the number of large antral follicles (Table 5).

DISCUSSION

This paper reports a simple and rapid enzymatic technique for isolation of intact follicles from the hamster ovary. Collagenase has long been used in preparation of cells and tissues for in vitro studies (Cavanaugh et al., 1963; Lacy and Kostianovsky, 1967). Purified collagenase is unable to dissociate tissue rich in protein, such as the rat tail (Kono, 1969). However, effective dispersal occurs if collagenase is combined with a proteolytic enzyme such as trypsin. Grob (1964) was the first to use enzymatic dissociation to isolate mouse and rat follicles, and the best results were obtained with pronase. Numerous reports point out the detrimental side effects of pronase; e.g., effects on the glycoprotein cell coat (Poste, 1977) and on insulin receptors (Kono, 1969). The degree of damage depends on the duration of treatment, the concentration of enzyme, and the type of tissue.

Rabbit ovarian follicles have been isolated by using a crude collagenase preparation (Nicosia et al., 1975). In preliminary experiments we tried this method to disperse hamster follicles but the results were unsatisfactory, possibly due to different tissue composition of the hamster ovary. By trial and error, we developed the present enzymatic "cocktail" which readily dissociated the hamster ovary. Short exposure time (20 min) of ovarian fragments to small amounts of pronase (0.0025%), and subsequent protection of the follicles with bovine serum albumin permitted the recovery of large numbers of intact follicles at different stages of development without any morphological signs of abnormality. Moreover, in preliminary experiments all stages of follicular development—from primary to antral—show excellent ability to incorporate [^{3}H] thymidine over a 4-h incubation (Roy and Greenwald, unpublished).

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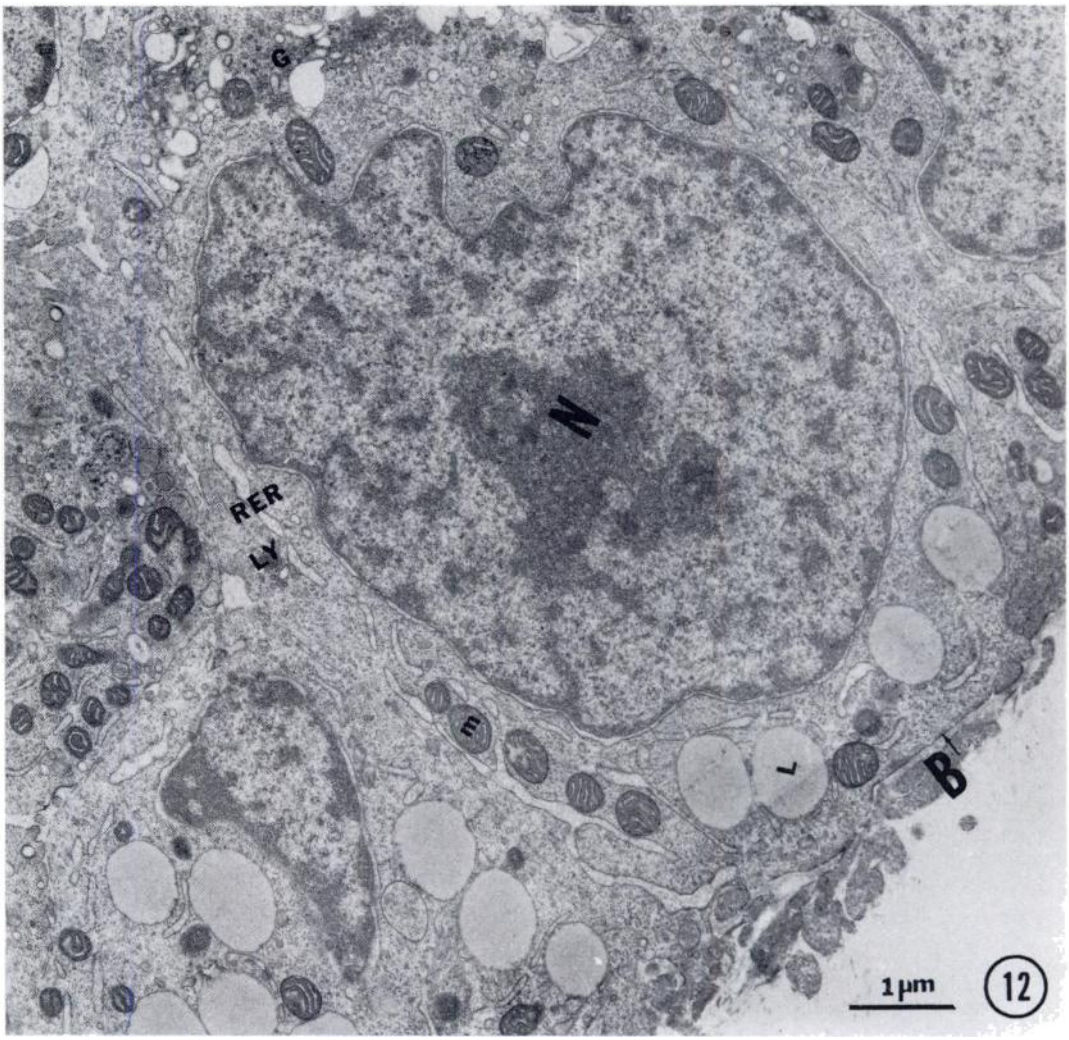

FIG. 12. Electron micrograph of a trilaminar (Stage 3) follicle showing normal morphological detail of isolated follicle. Note that there is no damage to the basement membrane (*B*). Large irregular nucleus (*N*), numerous well-defined mitochondria (*m*), rough endoplasmic reticulum (*RER*) and lysosomes (*LY*) are clearly visible. Presence of prominent subnuclear lipid droplets (*L*) indicates the possibility of potential steroidogenesis. X17,500.

In the present study, histological examination revealed that 74% of the atretic follicles were preantral stages with less than 5 layers of granulosa cells (Table 1). It is tempting to speculate that the development of the theca only when follicles have 7 to 8 layers of granulosa cells acts as a significant form of protection for the growing follicle. Occurrence of follicular degeneration due to insufficient development of thecal cells has been reported by Hisaw (1947) and by Peters (1978). The protection provided by the theca to the preantral follicle could be due to: *1*) its vascularity, which would enhance delivery of nutrients and other factors to the avascular granulosa cell compartment; *2*) its ability to bind luteinizing hormone (LH) which initially is limited to the theca (Ryan and Petro, 1966; Erickson and Ryan, 1976; Channing et al., 1980); *3*) its ability to synthesize androgens for export to the granulosa cells for conversion to estrogens; or *4*) its production of nonsteroidal factors needed for growth and differentiation of granulosa cells (Mondschein and Schomberg, 1981; St. Arnaud et al.,

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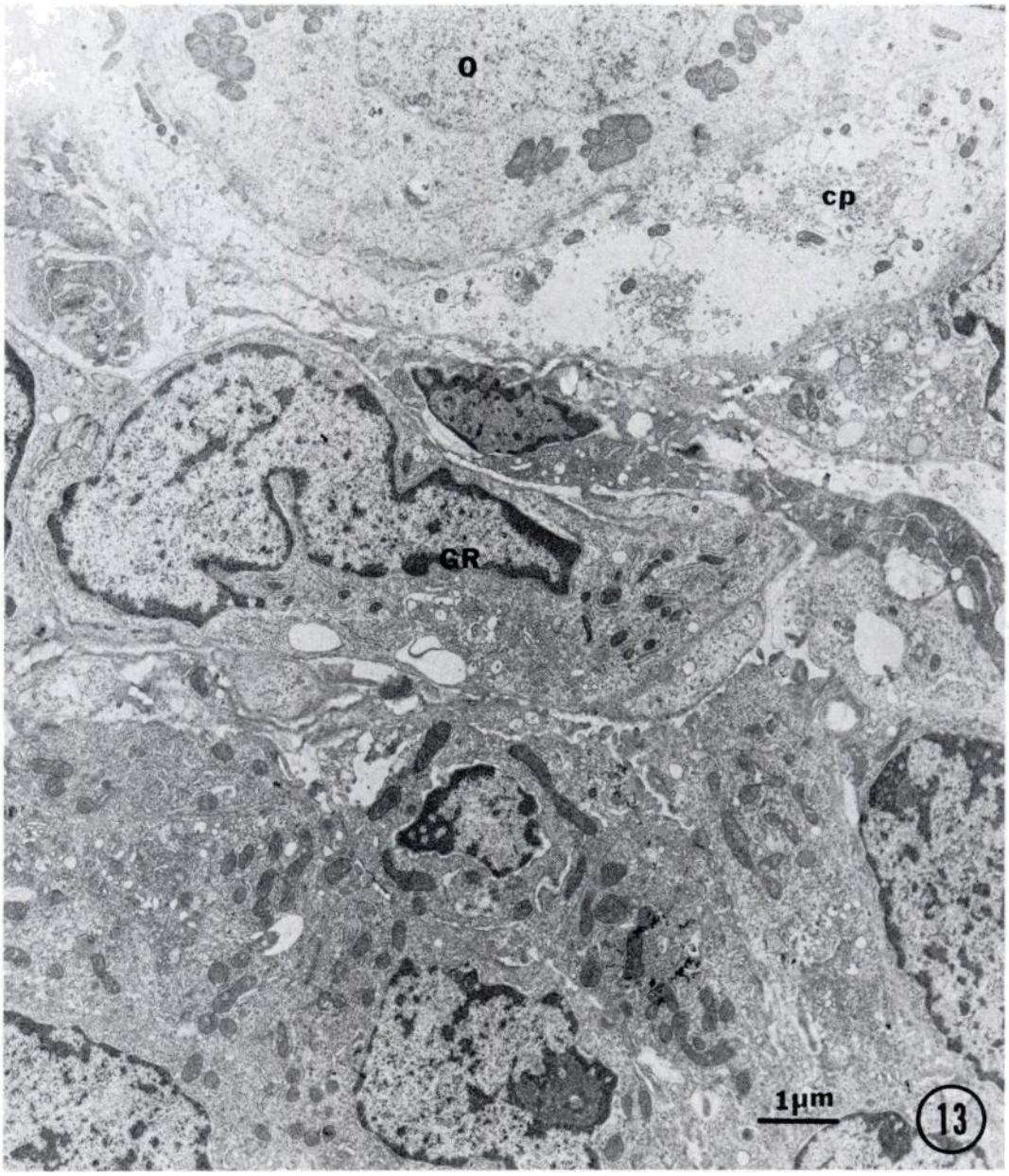

FIG. 13. Electron micrograph of a Stage 3 follicle showing healthy oocyte (*o*) and granulosa cells (*GR*). Numerous cumulus cell processes (*cp*) are seen traversing the zona pellucida. ×11,500.

1983). It is obvious that these are not mutually exclusive roles for the theca.

In this study, when observed by both light and electron microscopy, no trace of thecal cells was observed on small follicles with up to 6 layers of granulosa cells when the follicles were isolated by strictly mechanical agitation. In histological sections of hamster ovaries it is difficult to demonstrate the onset of thecal cell formation since fusiform, nonepithelioid cells accumulate in a whorl-like fashion in the vicinity of small follicles. It is unlikely that the short exposure to the enzymes could detach well-knit thecal cells from the follicles. Never-

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TABLE 2. Distribution according to size of dissected healthy follicles in the hamster ovary.

Stage of follicular development*	Range in diameter (μm)	Percent follicles†	Diameter of precalibrated pipette (μm)
1	28– 55‡	98.6	55
	56– 74	1.4	
2	29– 55	10.0	
	56– 92	82.0	92
	93–110	8.0	
3	75– 92	6.2	
	93–130	92.6	110
	131–148	1.2	
4	93–100	6.8	
	111–163	75.8	148
	149–225	24.0	
5	131–163	21.7	
	164–225	65.0	225
	241–370	13.3	
6	186–225	4.0	
	241–330	89.3	330
	331–370		
7	186–259	1.9	
	260–370	81.2	389
	371–445	16.9	
8	279–330	6.1	
	331–420	81.2	420
	420–550	12.4	
9	279–370	9.0	
	371–550	59.7	550
	>550		
10	390–550	17.2	
	>550	82.8	>550

*See Table 1 for classification of stages.

†Based on total of healthy and atretic follicles for each stage as listed in Table 1.

‡*Underlined* value is size range in which most follicles are present.

theless, we cannot rule out the possibility that the removal of loosely arranged cells around small follicles eliminates cells which may already in situ play a functional role.

Ultrastructural examination reveals normal cellular organization of the granulosa cells of the small follicles (Fig. 12). In small follicles, the granulosa cells extend projections towards the oocyte through the zona pellucida to intermix with microvilli formed by the oocyte plasma membrane (Weakley, 1966). We have also observed this anatomical link in small hamster follicles. The presence of follicle cells with irregular and indented nuclei, abundance of mitochondria and granular endoplasmic reticulum, lipid droplets and high nuclear-cytoplasm ratio in hamster ovarian follicles tally well with the characteristics of steroidogenic cells of the ovary as reported earlier (Albertini and Anderson, 1974). In the rabbit, Nicosia and coworkers (1975) have also described these features in small follicles. The alignment of lipid droplets at the basal nuclear region of peripheral granulosa cells provides strong evidence for

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TABLE 3. Follicular population on different days of the hamster estrous cycle*.

Day of cycle	Mean number of follicles ± SEM per animal in stage:†										Mean number follicles per animal
	1	2	3	4	5	6	7	8	9	10	
1–0900 h	99 ± 8	76 ± 9	57 ± 9	75 ± 20	99 ± 11	13 ± 3	8 ± 1	13 ± 2	2.5(2)	4 ± 2(4)^{fd}	460 ± 34
1–1500 h	111 ± 12	110 ± 12^{a}‡	87 ± 6^{a}‡	101 ± 9^{a}‡	91 ± 19	23 ± 3	8 ± 1	17 ± 2^{a}‡	7 ± 1^{ae}‡	2 ± 0.3(3)	548 ± 49
2–0900 h	74 ± 11	100 ± 11	56 ± 15	57 ± 12	109 ± 20	11 ± 1	6 ± 1^{d}	13 ± 2	12 ± 2^{e}	---	437 ± 42
2–1500 h	138 ± 21§	67 ± 13^{a}	67 ± 11	43 ± 5^{a}	119 ± 12	25 ± 2	6 ± 1	10 ± 1^{a}	21 ± 2^{c}‡	---	494 ± 30
3–0900 h	82 ± 9	80 ± 14	51 ± 5	79 ± 11	69 ± 20	15 ± 2	6 ± 1^{d}	8 ± 1	7 ± 1^{ef}	10 ± 1^{f}‡	424 ± 35
3–1500 h	139 ± 33	79 ± 5	61 ± 7	57 ± 5^{a}	77 ± 9^{b}	16 ± 1^{b}	6 ± 1	8 ± 2^{a}	5 ± 0.5^{e}	17 ± 1	471 ± 39
4–0900 h	114 ± 7	87 ± 11	59 ± 6	68 ± 8	90 ± 9	12 ± 1	11 ± 1^{d}‡	7 ± 1	3 ± 0.4^{ef}	14 ± 0.5^{d}‡	463 ± 30
4–1500 h	93 ± 21	96 ± 16	43 ± 5^{a}	58 ± 11^{a}	147 ± 22^{b}‡	27 ± 4^{b}‡	10 ± 2	4 ± 1^{a}	2 ± 0.2^{ca}	13 ± 1	492 ± 66

*There are six animals per group except for Day 1–1500 h (n=12). Follicles were collected by the second method as a separate enzymatic incubation for each animal killed at 0900 h and 1500 h.

†See Table 1 for definition of stages.

‡Values are significantly greater than values with the same *superscript letter*.

§Level of statistical significance=P<0.05. *Underlined numbers* are significantly different from the corresponding stage on the *same* day.

$^{a-c}$Indicate comparison of values at *1500 h* on each day of the cycle.

$^{d-f}$Refers to comparison at *0900 h* for each day of the cycle.

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TABLE 4. Effects of hypophysectomy (*H*) at 0900 h, Day 1 on subsequent follicular development*.

Treatment and day killed	Mean number of follicles ± SEM per animal in stage:									
	1	2	3	4	5	6	7	8	9	10
Intact Day 4—0900 h†	114 ± 7	87 ± 11[a]	59 ± 6	68 ± 8	90 ± 9[ab]	12 ± 1[ab]	11 ± 1[c]†	7 ± 1	3 ± 0.4	14 ± 1
H Day 4—0900 h	122 ± 13	126 ± 12[a]‡	75 ± 8	90 ± 17	180 ± 24[a]‡	25 ± 5[a]‡	3 ± 1(3)[c]	---	---	---
H Day 8—0900 h	91 ± 15	116 ± 10	80 ± 8	93 ± 9	124 ± 12[b]‡	33 ± 3[b]‡	---	---	---	---

*There are 6 animals per group.

†Data from Table 3.

‡,[a-c]The value indicated by a ‡ is significantly greater ($P<0.05$) than values with the same *superscript letter*.

TABLE 5. Effects of unilateral ovariectomy (ULO) at 0900 h, Day 3 on subsequent follicular development*.

Treatment and day killed	Mean number of follicles ± SEM per ovary in stage									
	1	2	3	4	5	6	7	8	9	10
Intact Day 3—0900 h	73 ± 11[a]	64 ± 10	22 ± 2[a]	67 ± 8[b]†	34 ± 6[ca]	15 ± 2	4 ± 1	6 ± 1[a]	5 ± 1	5 ± 1[a]
ULO Day 3—1500 h	81 ± 9	51 ± 5	25 ± 2[a]	23 ± 1[b]	83 ± 8[c]†	12 ± 2[a]	6 ± 0.4	6 ± 0.6[a]	4 ± 0.4	7 ± 0.7[a]
ULO Day 4—0900 h	106 ± 11[a]†	51 ± 5	33 ± 4[a]†	24 ± 5[b]	94 ± 10[a]†	20 ± 3[a]†	7 ± 1	10 ± 1[a]†	4 ± 1	12 ± 0.4[a]†

*There are 6 animals per group.

†,[a-c]The value indicated by a † is significantly greater ($P<0.05$) than values with the same *superscript letter*.

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potential steroidogenesis. Numerous autoradiographic studies on ovaries of different species with radiolabeled follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG)/LH have demonstrated that hormone binding on the surface of peripheral cells is much more than that of the cells lying further inside of the follicles (Midgley, 1973; Bortolussi et al., 1977, 1979; Oxberry and Greenwald, 1982). This is understandable in light of the fact that such cells are in direct contact with surrounding thecal or stromal cells, and hence are the first to be exposed to gonadotropic or other stimuli. Therefore, it is possible that lipid droplets in peripheral granulosa cells are utilized for steroidogenesis in the course of follicular development. Indeed, primary follicles from the rabbit ovary contain measurable amounts of estrogens (26.6 pg) and progesterone (24.2 pg) (Nicosia et al., 1975). Similarly, hamster follicles with three layers of granulosa cells produce large amounts of progesterone after incubation for 2 h with 25 ng FSH (basal: 180 ± 52 vs. 381 ± 18 pg/μg DNA; Roy and Greenwald, unpublished).

Greenwald (1974) published a detailed report on the population of hamster ovarian follicles during the estrous cycle. In our initial procedure we obtained a good number of follicles and the later use of mechanical agitation increased the yield severalfold (Table 3). Nevertheless, the follicle population in each group and on each day of the estrous cycle is markedly less than those reported from the histological study. The reason for this discrepancy could well be due to the application of several steps in the enzymatic procedure; such loss is quite common in all methods of cell isolation. Our study, however, shows similar trends in folliculogenesis on different days of the hamster cycle. The average number of follicles remains virtually the same on each day of the cycle. The most dramatic changes involve the subpopulation of antral follicles (Table 3). On Day 1, the larger ones are atretic, and intermediate sized antral follicles first appear on Day 2 and are consequently succeeded by large Graafian follicles on Day 3. Note that on Day 4 the average number of large antral follicles, 14 ± 0.5 per animal, is comparable to the ultimate number of ovulations.

The method, therefore, lends itself to assessing changes in follicular numbers and growth after various procedures. Thus, after hypophysectomy, the number of follicles in Stages 1–4 are quite similar to the profile in intact hamsters, suggesting that this represents a basal rate of proliferation of follicles which is unaffected by hormonal levels. On the other hand, after hypophysectomy significantly more large preantral follicles accumulated in Stages 5 and 6 than in the ovaries of intact animals (Table 4). This was not observed in a quantitative histological study in which only *healthy* follicles were counted (Moore and Greenwald, 1974). It therefore appears likely that the stockpiling of follicles at Stages 5 and 6 represents the accumulation of large numbers of atretic follicles which have been blocked from further development by the anhormonal environment of the hypophysectomized animal.

In the experiment involving the effects of unilateral ovariectomy (ULO) at Day 3 on follicular kinetics, we were guided by the results of two previous studies: *1*) an acute but transient increase in serum FSH begins by 4 h after ULO (Bast and Greenwald, 1977); and *2*) follicles with 6–7 layers of granulosa cells are recruited by 4 h after ULO (Chiras and Greenwald, 1978). The present results agree quite well with these findings in that 6 h after ULO, follicles had begun to migrate out of Stage 4 and shifted into Stage 5 (Table 5). Thus, Stages 4 and 5 follicles were the first to be affected by the altered hormone patterns elicited by ULO. The chronic long-term effects of ULO were reflected by 0900 h on Day 4 in a significantly greater number of follicles in Stages 1, 3, 5, 6, 8 and 10 compared to the intact animal at Day 3, 0900 h (Table 5). These changes are compatible with long-term actions of elevated levels of FSH. There is a discrepancy between follicular numbers per pair of ovaries (Table 3) versus the results of the ULO experiment where follicles are expressed per ovary (Table 5). The data in Table 5 was derived differently because it was part of a study involving [^{3}H] thymidine incorporation which necessitates the use of two ovaries to measure detectable levels. Hence for all groups in Table 5, follicles were pooled from two ovaries, each from a different animal, and the number of follicles per stage was then divided by 2 and results expressed as follicles per ovary. Another possible factor accounting for the discrepancy is the age of animals, which correlates with body weight. The hamsters used in the ULO experiments were considerably larger and this could skew the follicular population differently from animals weighing 100–110 g, which constituted the groups shown in Table 3.

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