Quantitative analysis of in-vitro incorporation of [³H]thymidine into hamster follicles during the oestrous cycle*

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Summary. Follicles were isolated from hamster ovaries at 09:00 h and 15:00 h on each of the 4 days of the oestrous cycle (Day 1 = oestrus; Day 4 = pro-oestrus) by microdissection and by a mixture of enzymes and classified into 10 stages with pre-calibrated pipettes (stage 1 = preantral follicles with 1 layer of granulosa cells; stage 10 = preovulatory antral follicles). The follicles at each stage were incubated for 4 h with [³H]thymidine with incorporation expressed per µg follicular DNA or per follicle. A significant increase in thymidine per follicle occurred at 15:00 h on Days 1 and 3 of the cycle from stage 2 (bilaminar follicle) to stage 6 (7–8 layers granulosa cells plus theca). When expressed as thymidine per follicle or µg DNA, there was a significant increase in incorporation for stages 1–4 (4 layers granulosa cells) on Day 4 at 15:00 h compared to 09:00 h, presumably as a consequence of the preovulatory increase in gonadotrophins. Follicles in stages 5 to 8 (preantral follicles with 5 or more layers of granulosa cells to small antral follicles), from which the next set of ovulatory follicles will be selected, did not show a significant peak in incorporation per µg DNA until Day 1 at 09:00 and 15:00 h when the second increase in FSH is in progress. DNA synthesis was similarly sustained throughout Day 1 for stage 1–4 follicles. These results suggest that periovulatory changes in FSH and LH, directly or indirectly, are not only responsible for ovulation and the recruitment of the next set of follicles destined to ovulate but also stimulate DNA replication in smaller follicles which develop over the course of several cycles before they ovulate or become atretic.

Introduction

Development from primordial to antral follicles in the mammalian ovary requires successive mitosis of granulosa cells and their subsequent differentiation. Folliculogenesis has been studied by many investigators using histological and autoradiographic techniques (Pederson, 1972; Hirshfield, 1984). Chiras & Greenwald (1980) evaluated follicular kinetics in the hamster ovary by injecting [³H]thymidine with autoradiography as the endpoint. Sheela Rani & Moudgal (1977) provided quantitative evidence for the in-vitro rate of incorporation of [³H]thymidine into the non-luteal ovarian compartment throughout the hamster oestrous cycle. However, their study did not deal with the growth potential of follicles at different stages of development. For this purpose, we have recently developed a method to isolate enzymically preantral follicles from the hamster ovary at defined states of development (Roy & Greenwald, 1985).

Since follicles in different stages show different developmental patterns (Chiras & Greenwald, 1977) and no quantitative biochemical data on follicular kinetics are available, the present investigation was designed to address two basic questions: (1) during the oestrous cycle do hamster

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ovarian follicles show differential growth potentials in terms of DNA synthesis? and (2) at what stage(s) of follicular development is DNA synthesis influenced by periovulatory changes in hormone concentrations? For this purpose [^3H]thymidine was used to monitor in-vitro DNA synthesis as an index of cell multiplication in follicles for each of the 4 days of the oestrous cycle.

Materials and Methods

[6- ^3H]Thymidine (sp. act. 23.6–23.9 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL), non-radioactive thymidine, collagenase, DNase, pronase, calf thymus DNA and bisbenzimidazole (Hoechst dye 33258) were procured from Sigma Chemical Co. (St Louis, MO). All other chemicals obtained from various commercial sources were analytical grade.

Isolation of follicles

Cyclic golden hamsters (*Mesocricetus auratus*) maintained under 14 h light and 10 h dark were used (lights on 05:00 h). Animals were decapitated at 09:00 h and 15:00 h on each day of the oestrous cycle (Day 1 = oestrus, the morning of ovulation associated with a copious vaginal discharge; Day 4 = pro-oestrus). For each animal, corpora lutea and antral follicles were dissected free of connective tissue and the remaining ovarian fragments were then digested with a mixture of collagenase, DNase and pronase as described previously (Roy & Greenwald, 1985). Briefly, minced ovaries (paired from each animal) were incubated with enzymes at 37°C for 20 min in a metabolic shaker with gentle mechanical agitation with a Pasteur pipette at 10-min intervals; the supernatant was discarded after centrifugation at 54 g at 4°C and the follicles resuspended in Ca^{2+} - and Mg^{2+} -free Krebs–Ringer with Hepes and EGTA, pH 7.0 (KRHG). Follicles were collected with precalibrated micropipettes and the different developmental stages were classified as follows: Stage 1 (< 55 μm in diameter with 1 layer of granulosa cells); Stage 2 (56–92 μm with 2 layers of granulosa cells); Stage 3 (93–110 μm with 3 layers of granulosa cells); Stage 4 (111–148 μm with 4 layers of granulosa cells); Stage 5 (149–222 μm with 5–6 layers of granulosa cells and developing thecal cells); Stage 6 (223–330 μm with 7–8 layers of granulosa cells and a well developed theca); Stage 7 (331–389 μm with incipient formation of an antral cavity); Stage 8 (390–420 μm with small antral cavity); Stage 9 (421–550 μm with intermediate antral cavity); Stage 10 (> 550 μm with large antral cavity, i.e. preovulatory follicle).

Incubation of follicles

After sorting the follicles into the 10 stages, they were rinsed well in fresh KRHG solution with 1% BSA, the number per stage counted and then incubated in 1 ml KRHG with BSA in disposable polystyrene tubes without capping for 4 h at 37°C in a Dubnoff metabolic shaker in the presence of 0.5 μCi [^3H]thymidine. The ranges in number of follicles incubated per tube were: Stage 1: 74–113; Stage 2: 78–87; Stage 3: 51–59; Stage 4: 57–79; Stage 5: 69–109; Stage 6: 12–15; Stage 7: 6–11; Stage 8: 7–13; Stage 9: 9–12; Stage 10: 10–14. The incubation was terminated by adding 50 μg unlabelled thymidine and placing the tubes in an ice-bath. The tubes were centrifuged at 2300 g for 10 min at 4°C to remove the supernatant, the follicles were rinsed twice with DNA assay buffer, pH 7.0 (described below) and finally suspended in 300 μl assay buffer and stored frozen at –20°C until assayed for DNA and incorporation of radioactivity. Samples of each time period for all 4 days of the oestrous cycle were assayed simultaneously to avoid interassay variation.

Determination of in-vitro incorporation of [^3H]thymidine into follicular DNA

Follicles were sonicated with a Kontes micro-ultrasonic cell disruptor with power setting at 5.2 W for 10 sec. The sonicate (100 μl) was co-precipitated with 100 μl 0.05% BSA with an equal

volume of 10% ice-cold trichloroacetic acid (TCA) for 10 min and centrifuged at 2300 *g* for 10 min; the supernatant was removed and saved. The pellet was washed once with 5% TCA and the two supernatants were pooled to count radioactivity in the acid-soluble fraction to determine the internal pool of [^3H]thymidine. The acid-insoluble fraction was dissolved in 300 μl 1 *N*- NH_4OH and radioactivity was counted in a Packard Tricarb liquid scintillation spectrometer with 65% efficiency for tritium using a xylene-based scintillation cocktail (3A70) with a programme which corrected for quenching. In preliminary experiments, Stage 7 follicles (8 large preantral follicles/tube) were incubated in the presence of 0.5 μCi [^3H]thymidine for 0–5 h to determine whether the amount of radionuclide was sufficient to maintain linear incorporation. The results were expressed as pg [^3H]thymidine incorporated per μg follicular DNA or fg [^3H]thymidine per follicle. Incorporation of [^3H]thymidine in the acid-precipitable fraction of Stage 7 follicles occurred linearly over a period of 5 h, whereas the acid-soluble fraction (the internal pool of [^3H]thymidine) reached a plateau by 4 h, thus indicating that 0.5 μCi [^3H]thymidine was sufficient for continuous labelling of follicular DNA synthesized during the period of in-vitro incubation. Furthermore, 10% TCA at 4°C did not remove any ^3H from [^3H]colicin E1 DNA.

The pattern of acid-soluble radioactivity in follicles of Stages 1–10 showed no correlative changes with the specific incorporation of [^3H]thymidine into DNA throughout the oestrous cycle (data not shown). This indicates, therefore, that the increases in incorporation of [^3H]thymidine into DNA resulted from changes in the rate of DNA replication in follicular cells rather than resulting from differences in specific activity in the intracellular radionuclide pool.

Determination of DNA

Follicular DNA content was measured by a microfluorometric method (Downs & Wilfinger, 1983) with modifications adapted to our study. The method uses a fluorochrome, bisbenzimidazole, which binds with high affinity to the large grooves of the A–T base pairs of the DNA duplex. Our modifications did not compromise the sensitivity of the method which was 5 ng calf thymus DNA or equivalent per tube.

Aliquants of follicular sonicate were co-precipitated with 0.05% BSA by equal volumes of 10% ice-cold TCA in 10 $\times$ 75 mm siliconized glass tubes for 10 min on ice, centrifuged at 2300 *g* for 10 min and the supernatant removed immediately to prevent any damage to double-stranded DNA by overexposure to acid. The pellet was dissolved and DNA was extracted with 10 μl 0.2% Triton X-100 in 1 *N*- NH_4OH at 37°C for 10 min with shaking. The DNA assay buffer consisted of 100 mM-NaCl, 10 mM-EDTA and 10 mM-Tris, pH 7.0. To each standard and unknown tube, 1.5 ml assay buffer containing 0.5 μl bisbenzimidazole (200 $\mu\text{g}/\text{ml}$ H_2O)/ml assay buffer were added and fluorescence was recorded at 350 nm excitation and 455 nm emission wavelength in a Perkin–Elmer recording spectrofluorometer after warming the tubes to 30°C.

A series of standards ranging from 10 to 200 ng per tube prepared from a stock solution of calf thymus DNA (50 $\mu\text{g}/\text{ml}$ H_2O , calibrated spectrophotometrically) were included in each assay to avoid any variation in DNA estimation due to fluctuations in sensitivity of the fluorometer. In preliminary experiments, there was no significant difference in whole ovarian DNA content estimated by the method originally described by Downs & Wilfinger (1983) and the present modifications.

In another experiment the DNA content of hamster granulosa cells was estimated to assess the number of cells in ovarian follicles at different developmental stages using the diphenylamine method of Burton (1956) and our modified microfluorometric method. Six adult female hamsters were killed at pro-oestrus (Day 4) at 09:00 h and ovaries were cleaned of fat and washed with fresh KRHG without Ca^{2+} and Mg^{2+} . Granulosa cells were collected from large preovulatory follicles (Stage 10) by puncturing them with a needle, the cells were washed twice with fresh medium and counted in a haemocytometer after resuspension in 1.4 ml medium. Cells from individual animals were handled separately. Cells were sonicated by a Kontes ultrasonic cell disruptor and 10 μl sonicate in duplicate were used to measure DNA fluorometrically. Similarly, residual sonicate was

Table 1. DNA content (ng/follicle) of hamster follicles at different stages of development throughout the oestrous cycle

Cycle		Stage of follicle									
Day	Time (h)	1	2	3	4	5	6	7	8	9	10
1	09:00	0.8 ± 0.1	3 ± 0.6	5 ± 1 ^a	6 ± 1 ^a	7 ± 2 ^a	34 ± 4	119 ± 12	198 ± 24 ^{ab}	—	—
1	15:00	0.4 ± 0.04	2 ± 0.2	5 ± 0.3	8 ± 1	16 ± 1 ^{**}	55 ± 4	138 ± 13	243 ± 18 ^{xy}	—	—
2	09:00	0.6 ± 0.1	3 ± 0.2	6 ± 0.4 ^b	12 ± 1 ^{abc*}	13 ± 2 ^{ab}	80 ± 15 [*]	169 ± 20 [*]	329 ± 21 ^a	510 ± 39	—
2	15:00	0.2 ± 0.01	2 ± 0.3	5 ± 0.5	6 ± 1 ^x	10 ± 1 ^x	37 ± 9	108 ± 17 ^{xy}	266 ± 12 ^b	373 ± 37	—
3	09:00	0.6 ± 0.05	2 ± 0.2	3 ± 0.3 ^a	3 ± 0.6 ^b	5 ± 1 ^b	51 ± 5	151 ± 12	376 ± 49 ^b	748 ± 70	835 ± 76 ^{**}
3	15:00	0.3 ± 0.03	3 ± 0.4	6 ± 0.4	11 ± 1 ^{**}	15 ± 1 [*]	74 ± 4 [*]	174 ± 19 ^x	370 ± 33 ^x	586 ± 34	552 ± 43 ^x
4	09:00	0.6 ± 0.1	2 ± 0.2	4 ± 1 ^b	5 ± 1 ^c	7 ± 2	79 ± 2	137 ± 14	266 ± 37	918 ± 151	550 ± 86 ^a
4	15:00	0.6 ± 0.1	2 ± 0.3	5 ± 0.5	6 ± 1	12 ± 2	74 ± 12	201 ± 12 ^{xy*}	410 ± 66 ^{xy*}	564 ± 47	916 ± 64 ^{**}

Values are mean ± s.e.m. for 6 animals in each group except on Day 1 at 09:00 and 15:00 h (N = 12).
For each follicle stage, values with similar superscript letters differ significantly ($P < 0.05$): a,b,c, for values at 09:00 h and x,y for values at 15:00 h.
*Values that differ significantly ($P < 0.05$) from those at 09:00 or 15:00 h on that day.

used in duplicate for estimating DNA by Burton's method. Protein and DNA were precipitated by adding an equal volume of 10% ice-cold TCA for 10 min at 4°C. Pellets were extracted by 300 µl 0.5 N-HClO₄ at 90°C for 30 min and 250 µl aliquants were reacted with diphenylamine reagent for spectrophotometric assay of DNA.

The results were assessed by analysis of variance by means of a Biomedical Data Package (BMDP) programme and Duncan's multiple range test. The level of statistical significance was at 5%.

Results

There were no significant differences in DNA content of follicles at Stages 1, 2 and 9 throughout the cycle (Table 1). On Day 1, only Stage 5 follicles differed in DNA content between the morning and afternoon. On Day 2, DNA values at 09:00 h were higher than at 15:00 h for follicles of Stages 4, 6 and 7; Stage 5 follicles showed a significant increase from the value at Day 1, 09:00 h. For Stages 3–6, DNA increased markedly on Day 3 at 15:00 h compared to 09:00 h, but for Stage 10 follicles DNA dropped at 15:00 h. An increase in DNA content occurred at 15:00 h on Day 4 for follicles at Stages 7, 8 and 10. The DNA content of granulosa cells estimated by spectrophotometric and fluorometric methods were similar (12.8 ± 0.7 and 12.5 ± 0.6 pg/cell), further suggesting the validity of our modified method. The range in cell numbers in hamster follicles at Stages 1–10 during the oestrous cycle, as calculated from cellular DNA content, is shown in Table 2.

Incorporation of [³H]thymidine into follicular DNA increased significantly in follicles of Stages 1–4 on Day 4, 15:00 h, after the FSH and LH surges (Table 3). Moreover, for Stages 6–9 a marked but not significant rise was also observed on Day 4, 15:00 h. It was not until Day 1, 09:00 h and 15:00 h, that a significant and sustained increase in [³H]thymidine incorporation occurred in follicles of Stages 5–8 and for the smaller follicles (Stages 1–4) as well. From Stage 2 onwards there was a trend for increased follicular thymidine incorporation on each day at 15:00 h with the exception of Stage 2 on Day 1 and Stages 5 and 9 on Days 1 and 2, respectively. In general, the amount of [³H]thymidine incorporated into DNA in follicles at Stages 5–10 was lower compared to the smaller stages, although the pattern of incorporation was similar to that observed for the smaller stages.

When [³H]thymidine incorporation was expressed per follicle, the pattern was almost identical (Fig. 1). Stage 1 follicles showed a drop in incorporation at 15:00 h and a rise at 09:00 h on Days 2 and 3, with a sharp increase on Day 4, 15:00 h. In contrast, Stage 2 follicles showed a daily rise at

Table 2. DNA content (estimated fluorometrically to be 12.5 pg/granulosa cell) related to cell numbers in hamster follicles at different stages of development throughout the oestrous cycle

Stages	Definition of stages	Range in DNA content (ng/follicle)	Estimated number of cells (range)
1	1 layer of granulosa cells	0.2–0.8	16–64
2	2 layers of granulosa cells	2–3	160–240
3	3 layers of granulosa cells	3–6	240–480
4	4 layers of granulosa cells	3–12	240–960
5	5–6 layers of granulosa cells and start of thecal formation	5–16	400–1 280
6	7–8 layers of granulosa and thecal cells	34–80	2 720–6 400
7	Beginning of antrum	108–201	8 640–16 080
8	Small antral follicle	198–410	15 840–32 800
9	Intermediate antral follicle	373–918	29 840–73 440
10	Preovulatory follicle	550–916	44 000–73 280

Table 3. Incorporation of [³H]thymidine into follicular DNA (pg [³H]thymidine/ μ g DNA per 4 h) at different stages of development during the 4 days of the hamster oestrous cycle

Cycle		Stage of follicle									
Day	Time (h)	1	2	3	4	5	6	7	8	9	10
1	09:00	74 $\pm$ 7 ^{a*}	51 $\pm$ 9 ^{abc}	33 $\pm$ 2 ^{abc}	23 $\pm$ 3	40 $\pm$ 3 ^{abc}	31 $\pm$ 5 ^{ab}	22 $\pm$ 2 ^a	19 $\pm$ 3 ^{abc}	—	—
1	15:00	43 $\pm$ 8 ^z	48 $\pm$ 9	48 $\pm$ 5 ^{yz}	43 $\pm$ 9 ^x	30 $\pm$ 4 ^{xyz}	29 $\pm$ 3 ^{yz}	35 $\pm$ 6 ^{yz}	16 $\pm$ 2 ^{yz}	—	—
2	09:00	55 $\pm$ 8 [*]	17 $\pm$ 2 ^a	16 $\pm$ 2 ^a	13 $\pm$ 2	13 $\pm$ 2 ^a	9 $\pm$ 1	13 $\pm$ 2	8 $\pm$ 0 ^{7a}	8 $\pm$ 2 ^{ab*}	—
2	15:00	33 $\pm$ 4 ^y	30 $\pm$ 3	23 $\pm$ 10 ^x	25 $\pm$ 6	3 $\pm$ 0 ^{2x}	11 $\pm$ 6 ^s	32 $\pm$ 7 ^{y*}	4 $\pm$ 0 ^{3x}	4 $\pm$ 1	—
3	09:00	64 $\pm$ 8	11 $\pm$ 3 ^b	11 $\pm$ 1 ^b	8 $\pm$ 2	9 $\pm$ 2 ^b	11 $\pm$ 1 ^a	11 $\pm$ 2	4 $\pm$ 1 ^b	3 $\pm$ 0 ^{5a}	4 $\pm$ 1
3	15:00	44 $\pm$ 4 ^x	38 $\pm$ 6	25 $\pm$ 5 ^y	20 $\pm$ 3 ^s	17 $\pm$ 3 ^y	16 $\pm$ 2 ^y	16 $\pm$ 1 ^s	6 $\pm$ 1 ^y	4 $\pm$ 0 ⁵	2 $\pm$ 0 ²
4	09:00	24 $\pm$ 2 ^a	11 $\pm$ 1 ^c	10 $\pm$ 1 ^c	8 $\pm$ 2	9 $\pm$ 1 ^c	5 $\pm$ 1 ^b	4 $\pm$ 1 ^a	1 $\pm$ 0 ^{4c}	0 ⁵ $\pm$ 0 ^{1b}	3 $\pm$ 0 ⁷
4	15:00	88 $\pm$ 4 ^{xyz*}	43 $\pm$ 3 [*]	49 $\pm$ 8 [*]	37 $\pm$ 4 [*]	11 $\pm$ 2 ^z	14 $\pm$ 3 ^c	7 $\pm$ 1 ^{yz}	4 $\pm$ 1 ^{z*}	4 $\pm$ 1 [*]	1 $\pm$ 0 ⁴

Values are mean $\pm$ s.e.m. for 6 animals in each group except on Day 1 at 09:00 and 15:00 h (N = 12).
For each follicle stage, values with similar superscript letters differ significantly ($P < 0.05$): a, b, c, for values at 09:00 h and x, y, z for values at 15:00 h.
*Values that differ significantly ($P < 0.05$) from those at 09:00 or 15:00 h on that day.

15:00 h. Incorporation of [3 H]thymidine into DNA of Stage 3 and 4 follicles showed a similar profile to that of Stage 2 follicles (Fig. 1). Incorporation was significantly elevated at 15:00 h on Days 1 and 3 and low at 09:00 h on these days with a steep fall on Day 3, 09:00 h. There was then a sharp increase at Day 4, 15:00 h. A significant increase in incorporation also occurred in Stages 5, 6 and 7 for Day 1, 15:00 h and Day 3, 15:00 h. Follicular incorporation of [3 H]thymidine increased abruptly at 15:00 h on Day 4 for Stages 8 and 9. The follicles destined to ovulate, Stage 10, did not show any appreciable change in incorporation once they appeared on Day 3, 09:00 h. Follicles at Stages 5–8 showed sustained rates of incorporation of [3 H]thymidine at 15:00 h on Day 4 and also on the next day.

Fig. 1. Incorporation of [3 H]thymidine into hamster ovarian follicles at Stages 1–10 on each day of the oestrous cycle. Follicles were isolated enzymically and incubated *in vitro* in the presence of [3 H]thymidine as described in the text. Values are mean $\pm$ s.e.m. *Values at 09:00 or 15:00 h significantly different ($P < 0.05$) from those at 09:00 or 15:00 h on the same day.

Discussion

The salient finding is that small preantral follicles (Stages 1–4) show a significant increase in [^3H]thymidine incorporation by 15:00 h on Day 4 when there is also a sudden, but not uniformly significant, increase in DNA synthesis in the larger follicles. It is not until 09:00 h and 15:00 h of oestrus (Day 1) that significant activity is evident for Stage 5–8 follicles (Table 3; Fig 1). These results agree quite well with our previous study in which autoradiography of healthy follicles was used as the endpoint (Chiras & Greenwald, 1980); the peak labelling index on Day 4 was in follicles with 7 or fewer layers of granulosa cells and on Day 1 in small follicles with 2–12 layers of granulosa cells. In the latter study follicles with 1 layer of granulosa cells were not counted. If anything, the pattern is easier to discern in the present study because of the reproducibility of the biochemical technique as attested by the small standard errors of the means for most of the groups.

This is the first time after [^3H]thymidine administration that a method other than autoradiography has been used to analyse follicular kinetics. The major advantage of the method is its ability to produce quantitative results. The separation of atretic from normal follicles before incubation with [^3H]thymidine would be useful, but does not seem to be possible. However, in a previous study, 2191 follicles were sectioned and histologically examined and 17% were found to be atretic (Roy & Greenwald, 1985). This is undoubtedly an overestimate of the number of atretic follicles since the presence of 4 or more pycnotic nuclei was used as the criterion of atresia and a large population of these follicles, in very early stages of atresia, would still be able to incorporate [^3H]thymidine as evidenced by a previous autoradiographic study (Hirshfield, 1984). The fact that the results agree so well when expressed as [^3H]thymidine incorporated per follicle or per μg DNA also contributes to our confidence that atresia does not confound the findings and the fraction of advanced atretic follicles is relatively constant throughout the cycle. Furthermore, the method of DNA estimation loses efficiency by 50–75% for the denatured DNA molecule (Kapusinski & Skoczylus, 1977) and would thus tend to minimize the contribution of advanced atretic follicles.

The observed profiles for [^3H]thymidine incorporation into the different stages of follicles correlate with periovulatory changes in serum concentrations of FSH and LH. For example, both gonadotrophins begin increasing on Day 4 at 13:00–14:00 h and peak at 16:00–17:00 h (Bast & Greenwald, 1974). Beginning at 22:00 h on Day 4, there is an increase in serum FSH, accompanied by low levels of LH, which is sustained throughout the afternoon of Day 1 (Siegel, Bast & Greenwald, 1976). The injection of phenobarbital at 13:00 h blocks the incorporation of [^3H]thymidine into Stage 1–9 follicles (S. K. Roy & G. S. Greenwald, unpublished). The possibility cannot be excluded that other hormones, e.g. steroids, insulin, growth hormone, thyroid stimulating hormone and growth factors, may play a role in regulating follicular development at pro-oestrus and oestrus. However, there is already considerable evidence to point to FSH and LH as the major controlling factors. For example, FSH stimulates mitosis of granulosa cells in the rat (Peluso & Steger, 1978) and causes follicular recruitment in the rat (Hoak & Schwartz, 1980) and in the hamster (Greenwald, 1975; Greenwald & Siegel, 1982). Eshkol & Pariente (1984) have shown that FSH significantly stimulates [^3H]thymidine incorporation in rat granulosa cells in culture while hCG has no effect.

Consistent with the results reported by Chiras & Greenwald (1980) our study provides evidence, albeit indirect, that the periovulatory rise in serum FSH concentration is responsible for regulating the growth of not only the large secondary follicles but also of the primary and small secondary follicles (Stages 1–4). In the hamster the development of preovulatory follicles from small primary follicles (2–3 layers of granulosa cells) requires about 20 days (Chiras & Greenwald, 1977). It is therefore reasonable to assume that smaller follicles need at least 5 successive exposures to periovulatory surges of hormones until they become antral follicles. In hamsters hypophysectomized on Day 1 of the cycle, 99% of the viable follicles have 5 or fewer layers of granulosa cells (Moore & Greenwald, 1974).

The lack of a significant increase in [^3H]thymidine incorporation into cellular DNA in large

preantral (Stages 5–7) and antral follicles (Stage 10) on Day 4, 15:00 h may be due to a direct inhibitory effect of rising concentration of serum LH on DNA synthesis. Chiras & Greenwald (1978) showed that, in hypophysectomized hamsters, LH alone decreased follicular growth and antagonized some of the effects of FSH. In the hypophysectomized hamster, FSH treatment considerably increases the labelling index and intensity in small Stage 1 follicles (2–3 layers of granulosa cells) while LH has no effect (Chiras & Greenwald, 1978). Topical autoradiography reveals that follicles up to Stage IV (8–10 layers of granulosa cells in the terminology used by Moore & Greenwald, 1974) on Day 1 contain FSH but no LH receptors on the granulosa cells (Oxberry & Greenwald, 1982). It is therefore unlikely that LH affects [³H]thymidine incorporation in follicles at Stages 1–4 on Day 4, 15:00 h, since they lack thecal cells which are the known sites for LH action; however, LH may affect Stages 5–10 which have a well defined thecal covering (Roy & Greenwald, 1985).

The increase in DNA values in follicles at Stages 7, 8 and 10 on Day 4, 15:00 h clearly indicate a rapid growth of follicles after the gonadotrophin surge. In our study we found that the DNA content of hamster granulosa cells on the morning of pro-oestrus was 12.5 ± 0.6 pg/cell. It is possible that DNA per granulosa cell might differ on different days of the cycle but there is no evidence of this. Based on the value of 12.5 pg DNA/cell for the follicles at each stage of development (Stages 1–10) there was a range of variation in the total number of cells (Table 2). This explains the variation in follicular diameters in each stage which was observed by Roy & Greenwald (1985).

The incorporation of [³H]thymidine into ovarian follicles in each stage, except for Stage 1, declined at 09:00 h and rose at 15:00 h on Days 1, 3 and 4 of the oestrous cycle. The significance or causes of these fluctuations are unknown, but they have also been observed when the entire non-luteal ovary was tested (Chiras & Greenwald, 1980). Serial bleeding of cyclic hamsters at 4-h intervals revealed peaks of serum progesterone on the afternoons of Days 1–3 in addition to the pro-oestrous surge (Ridley & Greenwald, 1975). It may be that in the hamster progesterone rather than oestrogen is an important mitogenic stimulus for growth of small preantral follicles. Follicular cells in the hamster ovary may follow a rhythm in the cell cycle with DNA synthesis (S-phase) at 15:00 h and G₂ or mitosis (M) at 09:00 h. Further work is needed to prove or disprove this interpretation and to establish definitively which hormones are responsible for the periovulatory shifts in this phase. The present application of a well established biochemical technique to the problem of follicular kinetics offers a new approach to determine the factors regulating follicular selection.

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