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Protein and RNA Synthesis in Follicles Isolated from Rabbit Ovaries[1] (38675)

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Several methods of evaluation have shown that coitus or gonadotropin injection leads to a wave of secretion and synthesis of progestins, androgens, and estrogens by the rabbit ovary (1–5). This increased secretion appears to be transitory, however, since blood and tissue steroids fall to virtually unmeasurable levels by the time of ovulation. It was the purpose of these experiments to determine whether this cessation extended throughout the general metabolism of the follicle in the period before ovulation or whether it was restricted only to the steroidogenic pathways. Accordingly, measurements have been made of the incorporation of precursors into protein and RNA by follicles obtained from mated rabbits. The studies have been extended to include an investigation of the action of LH, FSH, and cyclic AMP on the *in vitro* synthesis of protein and RNA by follicles.

Experimental Procedure. Chemicals. All solvents used were reagent grade and most were distilled prior to use. DL-{carboxy-^{14}C}methionine (6–8 mCi/mmole) and {2-^{14}C}uridine (55.5 mCi/mmole) were purchased from New England Nuclear and used without further purification. Adenosine 3′:5′-monophosphate (cyclic AMP), cycloheximide and actinomycin D were purchased from Calbiochem. Luteinizing hormone (NIH-LH-S16) and follicle stimulating hormone (NIH-FSH-S8) were obtained from the National Institutes of Health.

Animals. Sexually mature, white female rabbits of the New Zealand strain weighing 3–4 kg were used. Animals were housed in individual cages with compressed food and water available *ad libitum*. In some of the experiments, the females were mated to a vigorous buck rabbit; the success of mating was checked by examining vaginal washings microscopically for spermatozoa.

Preparation and incubation of tissues. Mated rabbits were sacrificed by a sharp blow to the head immediately after (0 hr) or 2 hr, 6 hr, or 12 hr after mating. Whole ovarian follicles were then dissected free from surrounding tissue as previously described (6). In the 12 hr postcoital group, only ovulated (hemorrhagic) follicles were selected. The follicles from both ovaries of a single rabbit were pooled and then divided equally between 2 or 4 incubation vessels containing 2 ml of Krebs–Ringer bicarbonate buffer and 10 μCi of either ^{14}C methionine or ^{14}C uridine. In some experiments, follicles were obtained from unmated rabbits and LH, FSH, or bovine serum albumin (BSA) for control was added to the incubation vessel to give a final concentration of 5 μg/ml. Cyclic AMP was added to give a final concentration of 20 μM in a specially modified buffer in which the amount of NaCl was reduced in order to maintain isotonicity. In some studies, cycloheximide or actinomycin D was added to give a final concentration of 10 μg/ml or 50 μg/ml respectively. Incubations were performed at 37° in an atmosphere of 95% O_2:5% CO_2, lasted 2 hr, and were terminated by quick freezing.

Protein purification and determination. After thawing, the incubation medium was decanted and the follicles washed twice with buffer to remove most of the adsorbed substrate. The follicles were homogenized in buffer, proteins were precipitated by the addition of 0.2 vol of ice cold 15% sulfosalicylic acid and the resulting precipitate collected by centrifugation. The supernatant was discarded and the precipitate dissolved in 0.1 M NaOH for reprecipitation with sulfosalicylic acid. The resulting protein

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pellet was defatted with washes of acetone, acetone/ether, ether, and then air dried. This residue was dissolved in 0.1 *M* NaOH and aliquots taken for determination of the amount of protein present and the ^{14}C methionine content. The mass of protein was determined in each sample colorimetrically by a modification of the method of Lowry *et al.* (7). Another aliquot of the protein solution was counted directly for ^{14}C methionine incorporation in a solubilizer for counting aqueous samples (Insta-Gel, Packard Instrument Company).

RNA isolation and determination. The ribonucleic acids were isolated and quantitated by the method of Fleck and Munro (8). Samples were thawed, the medium was decanted, and the follicles washed twice with buffer. Follicles were homogenized in buffer and the nucleic acids and proteins precipitated by the addition of 0.5 vol of ice cold 2.1 *M* perchloric acid. This precipitate was isolated centrifugally, washed twice and defatted. RNA was extracted by incubating the resulting residue in 1 ml of 0.6 *M* KOH for 1 hr at 37°. An aliquot of the extract was counted directly in Insta-Gel for ^{14}C uridine incorporation while the absorbency of another aliquot was measured spectrophotometrically at 260 nm. The μg of RNA released by the alkaline hydrolysis was calculated as previously described (9).

Determination of Radioactivity. Radioactivity was determined using a Beckman Liquid Scintillation Spectrometer, Model LS-230 with automatic external standardization; ^{14}C efficiency was approximately 50%.

Calculation of results and statistical analyses. Since follicles were obtained from the same animal for experimental and control incubations, the paired variable *t* test was used to test the significance of difference obtained. The results of the mated rabbit studies were analyzed by the unpaired variable *t* test.

Results. In an attempt to standardize the follicles used in these studies, the diameter of each follicle was measured with an ocular micrometer and no follicle with a diameter less than 1.1 mm was incubated. Based on multiple observations, the average diameter of the follicles used was 1.8 ± 0.03 mm (diameter of largest follicle was 2.8 mm) with an average of 97 ± 8.4 μg protein/follicle and 6.0 ± 0.29 μg RNA/follicle (all values expressed as mean ± standard error).

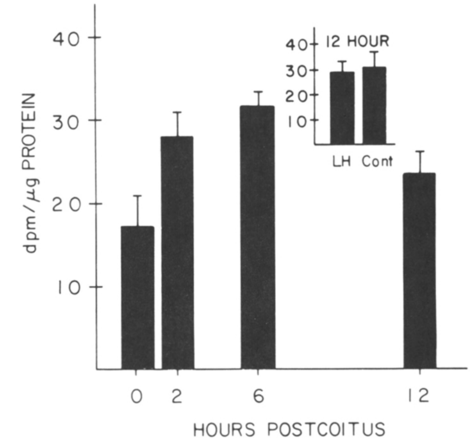

FIG. 1. The *in vitro* incorporation of ^{14}C methionine into proteins by follicles isolated 0, 2, 6, and 12 hr postcoitus. Each bar represents the mean of four to five incubations with brackets representing one standard error of the mean. Both the 2- and 6-hr postcoital groups are significantly greater than the 0 hr group ($P < 0.05$). INSERT. The results presented in the insert demonstrate the failure of LH (5 μg/ml) to stimulate ^{14}C methionine incorporation into protein by follicles isolated from 12-hr postcoital rabbits.

The data in Fig. 1 demonstrate the changes in ^{14}C methionine incorporation into protein by follicles isolated from mated does. Within 2 hr of mating, there was an increase over 0 hr levels in the incorporation of methionine into protein ($P < 0.05$). The significant increase was continued at 6 hr postcoitus, when incorporation rates were virtually twice the 0 hr level ($P < 0.01$). However, in ovulated follicles obtained 12 hr after mating, the incorporation rates declined to levels which were not different from those observed at 0 hr ($P < 0.1$). In order to determine whether the ovulated follicles retained their capacity to respond to gonadotropic stimulation, another group of 12 hr follicles was incubated with a stimulating dose of LH (5 μg/ml) or BSA as control. As can be seen in Fig. 1, these

TABLE I. THE INCORPORATION OF ^{14}C METHIONINE INTO PROTEIN BY OVARIAN FOLLICLES ISOLATED FROM UNMATED RABBITS.

A. The Effects of gonadotropins and cyclic AMP

Stimulating agent	Number of incubations	Specific activity (dpm/μg protein)
LH (5 μg/ml)	11	28.7 ± 3.1[a,b]
BSA	11	22.4 ± 2.7
FSH (5 μg/ml)	4	28.2 ± 3.1[b]
BSA	4	18.8 ± 2.3
FSH (5 μg/ml) + anti LH	6	23.0 ± 3.1[b]
BSA + anti LH	6	19.1 ± 2.3
Cyclic AMP (20 μM)	4	19.7 ± 3.4[b]
Control	4	13.1 ± 1.3

B. The Effect of cycloheximide (10 μg/ml)

Stimulating agent	Number of incubations	Specific activity (dpm/μg protein)
LH (5 μg/ml)	4	39.4 ± 4.5[b]
BSA	4	31.9 ± 3.2
LH + Cycloheximide	4	2.8 ± 1.0
BSA + Cycloheximide	4	2.2 ± 1.0

[a] Mean ± SEM.
[b] $P < 0.05$ when compared to paired control incubation by paired variable t test.

follicles incorporated no more ^{14}C methionine into protein than did the control follicles ($P > 0.3$).

In experiments to determine the gonadotropins responsible for these preovulatory changes, follicles were incubated with LH, FSH, or cyclic AMP, and ^{14}C methionine incorporation into protein measured. The results, presented in Table IA, demonstrate that LH, FSH, and cyclic AMP all significantly raised incorporation rates above paired control levels ($P < 0.05$). Since the FSH preparation (FSH-S8) used in these studies was reported to have 0.013 IU of LH activity/mg (10), another set of follicles was incubated with the FSH preparation in the presence of an antiserum prepared against ovine LH. The results of this incubation show that in the presence of the antibody to LH, FSH continued to increase the incorporation of the radioactive methionine into follicular protein. In another experiment, follicles were incubated with LH plus cycloheximide, an inhibitor of protein synthesis. As can be seen in Table IB, the inhibitor reduced methionine incorporation into precipitable protein to less than 10% of the level measured in the absence of the inhibitor. LH had no stimulatory effect in the presence of cycloheximide.

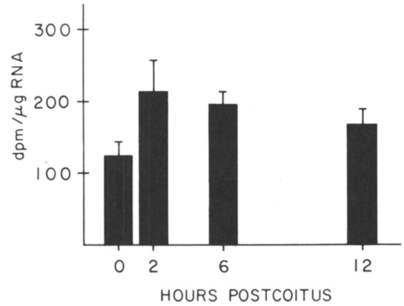

FIG. 2. The incorporation of ^{14}C uridine into RNA by follicles isolated 0, 2, 6, and 12 hr postcoitus. Each bar represents the mean of four to five separate incubations with brackets representing one standard error of the mean. When these data are analyzed by Student's t test, the 0 hr group is found to differ significantly from both 2- and 6-hr postcoital groups ($P < 0.05$).

Figure 2 presents the measurements made of ^{14}C uridine incorporation into RNA by follicles from animals mated 0, 2, 6, and 12 hr before sacrifice. Statistical analysis of these results demonstrates that incorporation rates were significantly elevated over unmated control levels at both 2 and 6 hr postcoitus ($P < 0.05$) while incorporation fell to near control levels by 12 hr after mating ($P < 0.2$). Using follicles from unmated rabbits, RNA synthesis could be stimulated by addition of exogenous gonadotropins to the incubations (Table IIA). Five μg of FSH/ml caused a marked increase in follicular RNA synthesis ($P < 0.05$); cyclic AMP likewise increased synthesis ($P < 0.05$) while LH failed to alter ^{14}C uridine incorporation into follicular RNA. Incubation of follicles with an inhibitor of RNA synthesis sharply reduced the incorporation of uridine into RNA both in the presence and absence of FSH (Table IIB).

Discussion. The results of the present experiments demonstrate that gonadotropins

TABLE II. THE INCORPORATION OF ^{14}C URIDINE INTO RNA BY OVARIAN FOLLICLES ISOLATED FROM UNMATED RABBITS.

A. The Effects of gonadotropins and cyclic AMP

Stimulating agent	Number of incubations	Specific activity (dpm/μg RNA)
LH (5 μg/ml)	13	533 ± 46[a]
BSA	13	508 ± 41
FSH (5 μg/ml)	11	479 ± 35[2]
BSA	11	367 ± 35
Cyclic AMP (20 μ*M*)	4	696 ± 49[b]
Control	4	504 ± 19

B. The Effect of Actinomycin D (50 μg/ml)

Stimulating agent	Number of incubations	Specific activity (dpm/μg RNA)
FSH (5 μg/ml)	4	392 ± 21[b]
BSA	4	293 ± 34
FSH + Actinomycin D	4	14 ± 4
BSA + Actinomycin D	4	11 ± 2

[a] Mean ± SEM.

[b] $P < 0.05$ when compared to paired control incubations by paired variable *t* test.

can stimulate follicular protein synthesis and RNA synthesis when added to the incubation vessel. In several previously reported studies, these gonadotropins were found to be ineffective *in vitro* (11–14). There are two differences between the present reported studies and those previously published in which the gonadotropins were ineffective. In the present study, whole follicles are isolated and incubated; follicles may be an intrinsically more responsive tissue than stromal or luteal tissue or sliced whole ovaries. Perhaps more importantly, however, the present experimental design utilizes paired incubations in which follicles from the same ovary were incubated either in the presence of the gonadotropin or with BSA. The use of paired incubations permits comparison of differences between treated and control follicles from the same animal and thereby eliminates the problem of variability between animals. Paired comparisons were not made in the previously published rat ovary studies and variability between animals may have contributed to the failure to demonstrate a gonadotropin effect on *in vitro* protein and RNA synthesis in these reports.

While the studies reported here demonstrate a stimulatory action of gonadotropins on follicular protein and RNA synthesis, they offer no insight as to how the hormones might exert this action. The studies of Jungmann and coworkers (15, 16) suggest that the gonadotropin may activate a cyclic AMP-dependent protein kinase. Preliminary results in this laboratory show that the transport of amino acids into the cells may also be involved. In these ongoing experiments, the *in vitro* transport of α aminoisobutyric acid (AIB) into follicles is stimulated by LH; the results of studies in which follicles are coincubated with LH plus cycloheximide show that the transport process is not sensitive to inhibitors of protein synthesis (Feit and Mills, unpublished observation). Since this protein synthetic inhibitor does effectively block methionine incorporation into protein (Table IB), it is possible that LH stimulates more than a single process in the rabbit follicle. This possibility is currently under investigation in our laboratory.

Immediately after mating in the rabbit, LH is released into the blood (2, 17, 18). This LH surge reaches a peak at about 2 hr after coitus and then falls rapidly to precoital levels at 4–6 hr; blood LH remains low through ovulation at 10–12 hr after mating (19). The postcoital incorporation of an amino acid into protein by rabbit ovarian follicles shows a similar transitory pattern except that the peak occurs at 6 hr rather than 2 hr (Fig. 1). Since LH stimulates ^{14}C methionine incorporation into follicular protein (Table IA), it is likely that the surge in blood LH levels is responsible for the transitory increase in follicular protein synthesis peaking at 6 hr postcoitus—possibly via a cyclic AMP intermediate.

The finding that follicular protein synthesis is increased after mating confirms the radioautographic studies of Pool and Lipner (20). These investigators demonstrated that the incorporation of labelled amino acids into rabbit thecal and granulosal cell protein was elevated 2 hr after coital stimulation. In their study, however, incorporation rates remained elevated through ovulation, while in the experiments reported here, incorporation of methionine into follicular protein fell to precoital levels in ovulated follicles (Fig. 1). Despite these studies, the nature or function of the protein synthesized in this period is not known. Protein synthesis is apparently required for ovulation in the rabbit because inhibitors will prevent ovulation if given by intrafollicular injection for up to 6 hr after mating (21). Rondell (22) has postulated the synthesis of an ovulatory enzyme, possibly proteolytic, in this preovulatory period.

General RNA synthesis in rabbit follicles also shows an increase following coital stimulation (Fig. 2). As was the case in follicular protein synthesis, uridine incorporation into RNA rose after mating but returned to control levels (0 hr) by 12 hr postcoitus. In their radioautographic study of follicular RNA synthesis, Pool and Lipner (20) reported this same temporal pattern with a preovulatory increase followed by a decline in radioactive uridine incorporation in ovulatory follicles. What agent stimulates this process *in vitro*, however, remains a question. RNA synthesis in isolated follicles proves to be insensitive to LH stimulation *in vitro*, while uridine incorporation is stimulated by the addition of FSH or cyclic AMP to the incubation (Table IIA). FSH is apparently not the sole stimulating agent of follicular RNA synthesis *in vivo*, however, since peripheral plasma levels of this gonadotropin have been reported to remain unchanged in the postcoital period (17). Other agents, possibly LH or steroids may synergize with FSH in the stimulation of follicular RNA synthesis *in vivo*.

Significance should be attached to the finding that follicular protein and RNA synthesis, as measured by ^{14}C precursor incorporation, falls to control levels at 12 hr postcoitus (Figs. 1 and 2). Furthermore, protein synthesis in the ovulated follicles is not responsive to stimulation; when exogenous LH is added to the incubation, no increase in precursor incorporation can be measured (Fig. 1). This same ovulatory cessation and refractoriness to exogenous gonadotropin has been reported for the follicular synthesis of steroids (23), cyclic AMP (24), and for steroidogenesis in sliced whole ovaries (1). It would appear that the preovulatory shutdown, previously observed only in the steroidogenic pathways, may extend throughout the general metabolism of the follicle in the period before ovulation.

Summary. The *in vitro* synthesis of protein and RNA in follicles from mated and unmated rabbits has been studied by measuring precursor incorporation. Radioactive methionine incorporation into follicular proteins showed a transitory rise following coitus but fell to precoital levels in ovulated follicles. LH, FSH, and cyclic AMP all exerted an acute stimulatory action on the incorporation of amino acids into proteins by follicles from unmated rabbits. Radioactive uridine incorporation into follicular RNA showed the same temporal pattern of postcoital rise followed by a decline in incorporation rates in the period around ovulation. In acute incubations, FSH and cyclic AMP stimulated RNA synthesis; LH was without acute action on this process. The results of these studies suggest that a wave of follicular protein and RNA synthesis precedes ovulation and may be partly related to the preovulatory surge in gonadotropins.

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