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Studies on follicular growth in the immature rat and hamster: Effect of a single injection of gonadotropin or estrogen on the rate of ^{3}H-thymidine incorporation into ovarian DNA *in vitro*

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Abstract. Initiation of follicular growth by specific hormonal stimuli in ovaries of immature rats and hamsters was studied by determining the rate of incorporation of ^{3}H-thymidine into ovarian DNA *in vitro*. Incorporation was considered as an index of DNA synthesis and cell multiplication.

A single injection of pregnant mare serum gonadotropin could thus maximally stimulate by 18 hr ^{3}H-thymidine incorporation into DNA of the ovary of immature hamsters. Neutralization of pregnant mare serum gonadotropin by an antiserum to ovine follicle stimulating hormone only during the initial 8-10 hr and not later could inhibit the increase in ^{3}H-thymidine incorporation *in vitro* observed at 18 hr, suggesting that the continued presence of gonadotropin stimulus was not necessary for this response. The other indices of follicular growth monitored such as ovarian weight, serum estradiol and uterine weight showed discernible increase at periods only after the above initial event.

A single injection of estrogen (diethyl stilbesterol or estradiol-17β) could similarly cause 18 hr later, a stimulation in the rate of incorporation of ^{3}H-thymidine into DNA *in vitro* in ovaries of immature rats. The presence of endogenous gonadotropins, however, was obligatory for observing this response to estrogen. Evidence in support of the above was two-fold: (i) administration of antiserum to follicle stimulating hormone or luteinizing hormone along with estrogen completely inhibited the increase in ^{3}H-thymidine incorporation into ovarian DNA *in vitro*; (ii) a radioimmunological measurement revealed following estrogen treatment, the presence of a higher concentration of endogenous follicle stimulating hormone in the ovary. Finally, administration of varying doses of ovine follicle stimulating hormone along with a constant dose of estrogen to immature rats produced a dose-dependent increment in the incorporation of ^{3}H-thymidine into ovarian DNA *in vitro*. These observations suggested the potentiality of this system for developing a sensitive bioassay for follicle stimulating hormone.

Keywords. Pregnant mare serum gonadotropin; follicle stimulating hormone; luteinizing hormone; estrogen; gonadotropin antisera; ovarian follicular growth; ^{3}H-thymidine incorporation into ovarian DNA; bioassay for follicle stimulating hormone.

1. Introduction

Development of the ovarian follicle from a preantral to an ovulable state primarily involves growth and successive multiplication of granulosa cells. The latter, in most studies, has been monitored by histological and autoradiographic techniques (Peters and Levy 1966; Pederson and Peters 1968; Pederson 1970; Schwartz 1974; Greenwald 1974). In a recent study using cycling hamsters (Sheela Rani and Moudgal 1977a), the rate of incorporation of ^{3}H-thymidine into ovarian DNA *in vitro* was used to monitor the initiation of follicular maturation during the estrous cycle. It was found that the ^{3}H-thymidine incorporation into DNA of the non-luteal ovarian

tissue follows a cyclical pattern and this could be correlated with follicular growth. Maximum incorporation during the cycle was seen at 12.00 hr of estrus and the specific stimulus fort his event was found to be the surge of follicle stimulating hormone (FSH) and luteinizing hormone (LH) occurring 18 hr earlier on the evening of proestrus (Sheela Rani and Moudgal 1977a).

The prepubertal or juvenile animal has been extensively used as a model system to examine the action of gonadotropins on the process of follicular growth. Induction of follicular maturation and ovulation in immature female rats treated with pregnant mare serum gonadotropin (PMSG) as originally described by Cole (1936) has been amply confirmed. Administration of a single appropriate dose of PMSG to immature rats is known to cause a surge in serum LH levels about 52–56 hr later (Costoff *et al* 1974), similar to the preovulatory surge of gonadotropins found in the adult cycling animal. Further, changes in plasma levels of estradiol, progesterone, 17 α-hydroxy-progesterone, testosterone, dihydrotestosterone, androstenedione, dehydroepiand-rosterone, LH, FSH, and estradiol receptors in pituitary and hypothalamus following administration of PMSG to immature rats have also been reported (Wilson *et al* 1974; Parker *et al* 1976; Sashida and Johnson 1976).

Another hormone, which has been implicated in stimulating follicular growth, is estrogen. Thus, administration of an estrogenic compound like diethyl stilbesterol for 2–4 days to hypophysectomized immature rats has been reported to result in an increase in the uptake of ^{3}H-thymidine as well as of ^{3}H-FSH by the ovaries *in vitro* (Goldenberg *et al* 1972). More recently, Richards *et al* (1976) have demonstrated that administration of estradiol to immature hypophysectomized rats increases the number of FSH receptors in granulosa cells, as judged by increase in binding of ^{125}I-labelled FSH *in vitro* to the granulosa cells isolated from these ovaries.

In the present study, using the rate of ^{3}H-thymidine incorporation into ovarian DNA *in vitro* as an index of DNA synthesis and cell division, and thus of initiation of follicular growth, we have studied the ability of two different types of hormones—a gonadotropin and a steroid—to stimulate this event in the ovaries of immature rats and hamsters. The effect of specific antisera to gonadotropins on this hormone-induced response in these two model systems has also been examined.

2. Materials and methods

2.1. *Animals*

Immature (25-27 day old) female golden hamsters (*Mesocricetus auratus*) and albino rats of our Institute colony were housed in well ventilated quarters provided with a regulated light: dark schedule of 14 : 10 hr. They were given pelleted food (Hindustan Lever Ltd., Bombay) and water *ad libitum*.

2.2. *Hormones and antisera*

PMSG and ovine FSH (NIH-FSH-S11) obtained from Sigma Chemical Co., USA and as a kind gift from the Hormone Distribution Program, NIH, Bethesda, USA, respectively were given dissolved in 0·9% NaCl. Diethyl stilbesterol (DES, from Sigma Chemical Co.,) or estradiol-17β (E_2, repurified Sigma sample) were initially

dissolved in a minimal volume of ethanol and diluted to the appropriate dose with 0·9% NaCl. All hormones were administered by the subcutaneous route.

The antisera to ovine FSH or LH(NIH preparations) used in the present study were raised in monkeys and rabbits respectively and were characterized to specificity prior to use as described elsewhere. The cross-reactivity of the antisera with the corresponding endogenous gonadotropin of the hamster and the rat has also been established earlier (Sheela Rani and Moudgal 1977b). These antisera were highly potent thus permitting the use of minimal effective doses.

2.3. *General methodology*

The experimental protocol describing the dose of hormone injected, the time of autopsy, the subsequent procedure followed, etc. are given under the section on results. At autopsy, the ovary and the uterus were quickly dissected, cleaned of adhering fat and weighed.

Determination of the rate of incorporation of ^{3}H-thymidine into ovarian DNA *in vitro* was done according to the method described by Sheela Rani and Moudgal (1977a). It involved incubation of ovarian minces in 1 ml Krebs Ringer bicarbonate buffer, pH 7·4 containing 50 mM Hepes (Sigma Chemical Co., USA) and 0·2% glucose with 1·0 μCi of ^{3}H-thymidine (New England Nuclear Corporation, Boston, sp. act. 20 Ci/mmole) for 4 hr at 37°C in a Dubnoff metabolic shaker. DNA was isolated from the ovarian tissue by cold perchloric acid precipitation followed by acid hydrolysis at 90°C for 20 min and the labelled thymidine incorporated into DNA was determined by liquid scintillation counting. The choice of the 4 hr incubation period was based on our earlier observation that under the conditions employed, the incorporation exhibited a linear pattern up to 4 hr indicating the viability of the tissue and the validity of the above system (Sheela Rani and Moudgal 1977a).

2.4. *Radioimmunoassay*

Estradiol level in serum samples was estimated by a radioimmunoassay as described earlier by Mukku and Moudgal (1975).

FSH level in the rat serum was estimated using the NIAMDD rat FSH radioimmunoassay (RIA) kit (obtained through the courtesy of NIAMDD, NIH, Bethesda) according to the method of Moudgal and Madhwaraj (1974). All the incubations in the RIA were done at 37°C. The samples or the standard hormone (NIAMDD-rat FSH-RP) were incubated first with the rat FSH antiserum for 12 hr followed by the addition of ^{125}I-labelled rat FSH [iodinated with carrier free Na ^{125}I (The Radiochemical Center, Amersham, UK) using chloromine-T essentially according to the procedure of Greenwood *et al* 1963]. At the end of the second 12 hr incubation, goat second antibody to rabbit gamma globulin was added to separate the bound and the free hormone and incubation continued for a further period of 12 hr.

FSH bound to the ovarian tissue of the rat was similarly estimated by the above procedure, using ovarian homogenates (~5–10 mg tissue per tube in 0·05 M phosphate-EDTA buffer, pH 7·4) instead of serum samples. The ability of this assay procedure to measure all the tissue-bound hormone has been validated (Sheela Rani 1977). For this purpose, after incubation of the tissue homogenate with the first antibody in the RIA, the tissue pellet and the supernatant were separated by centrifugation

and processed individually for FSH in the same RIA. While all the FSH were present in the supernatant, being bound by the first antibody used in the RIA, no FSH could be measured in the pellet. Further, little FSH could be measured by RIA in non-target organs for FSH such as uterus, lungs, etc.

3. Results

3.1. *Effect of administration of PMSG to immature hamsters*

A. Time-course of action: Administration of 15 IU of PMSG to 25 day-old female hamsters caused a 3-4 fold increase in the rate of ^{3}H-thymidine incorporation into ovarian DNA by 6 hr and it reached a peak by 18 hr, at which time a 5-fold increase was seen (figure 1). This rate of incorporation declined by 24 hr and returned to the basal level after 30 hr. The first significant increase in ovarian weight was seen around 12 hr and it progressively increased till 42 hr. The serum estradiol and the uterine weight showed discernible changes only 24 hr after administration of PMSG. The increase in serum estradiol (significant increase by 24 hr) preceded that of the uterine weight observed at 30 hr.

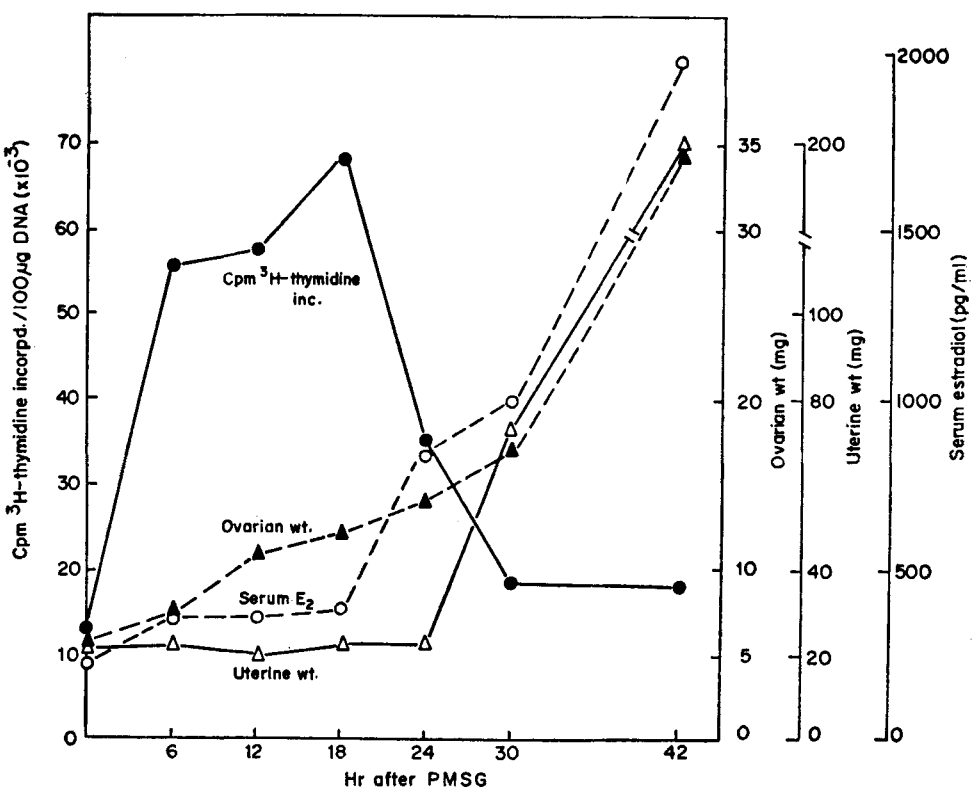

Figure 1. Effect of PMSG (15 IU) administered to immature hamsters. Time-course of ^{3}H-thymidine incorporation into ovarian DNA *in vitro* (●——●), ovarian wet weight (▲.....▲), serum estradiol (○——○) and uterine weight (▽——▽), Each point is a mean of values from 4 animals.

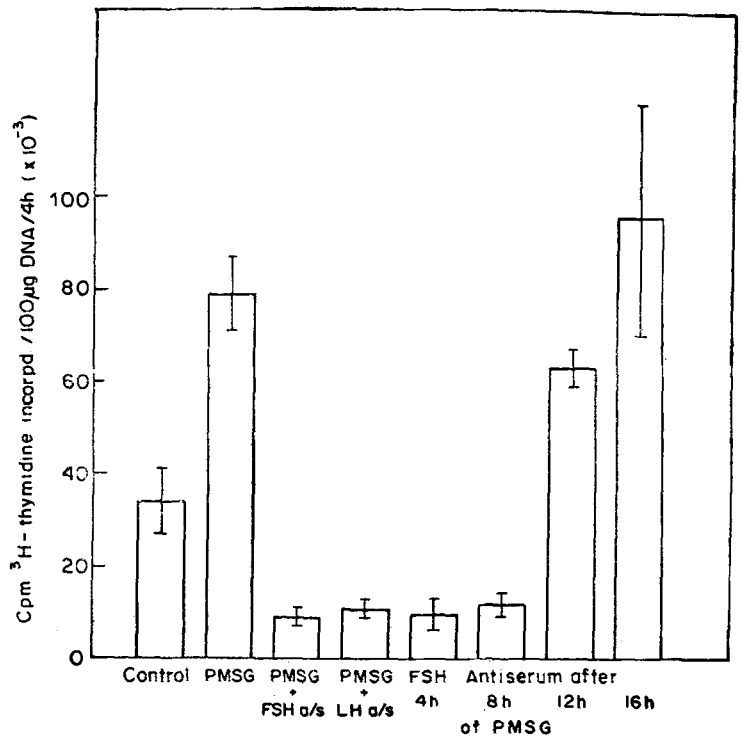

Figure 2. Effect of FSH antiserum (0·1 ml) at different times after PMSG on the rate of ^{3}H-thymidine incorporation into DNA *in vitro* of immature hamster ovary. One of the groups received LH a/s (0·2 ml) along with PMSG. All animals were killed 18 hr after the subcutaneous administration of 15 IU PMSG; the antisera were given intraperitoneally. Each bar represents a mean of values from 4 animals and the vertical lines represent the standard deviation.

B. Effect of FSH antiserum at various times after PMSG treatment on ^{3}H-thymidine incorporation into DNA of immature hamster ovary in vitro: Administration of either FSH or LH antiserum along with PMSG completely inhibited the stimulation to a level below the control values. This was perhaps due to the neutralization, in addition to PMSG, of endogenous FSH and LH as well (figure 2). In order to see upto what time this phenomenon is dependent on PMSG/FSH, antiserum to FSH was administered at various time intervals after PMSG. All animals were killed 18 hr after PMSG and the rate of ^{3}H-thymidine incorporation into ovarian DNA was measured. It was found that FSH antiserum administered 8 hr after PMSG could still inhibit the action of the latter. When FSH antiserum was injected 12 hr after PMSG, a small but statistically significant reduction in the ^{3}H-thymidine incorporation into DNA was observed. This suggested the presence of PMSG and/or FSH to be obligatory for the first 8–10 hr to effect an increase in incorporation of labelled thymidine into DNA, the event becoming independent of hormonal support thereafter.

3.2. *Effect of administration of estrogen and FSH on ^{3}H-thymidine incorporation into ovarian DNA in vitro amd ovarian FSH concentration in immature rats*

Groups of 25–27 day-old female rats were given the following treatments: 0·1 ml of 0·9% NaCl, 2 mg DES or 1·0 µg E_2 in 0·1 ml 0·9% NaCl; other groups of rats

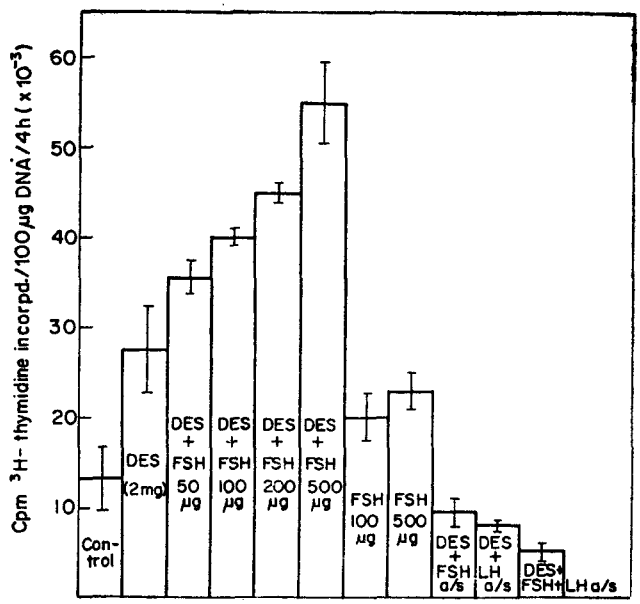

Figure 3. Effect of administration of DES (2 mg), either alone or with various doses of FSH (50-500 µg NIH-S11) to 25 day-old immature rats on ^{3}H-thymidine incorporation into ovarian DNA *in vitro*. Injections (in 0·9% NaCl), were given subcutaneously at separate sites on the back of the neck. The animals were killed 18 hr later and the rate of ^{3}H-thymidine incorporated into DNA of ovaries *in vitro* was determined. When antisera were given along with DES, they were injected intraperitoneally. Each column represents the mean value from 3 animals and the vertical lines represent the standard deviation.

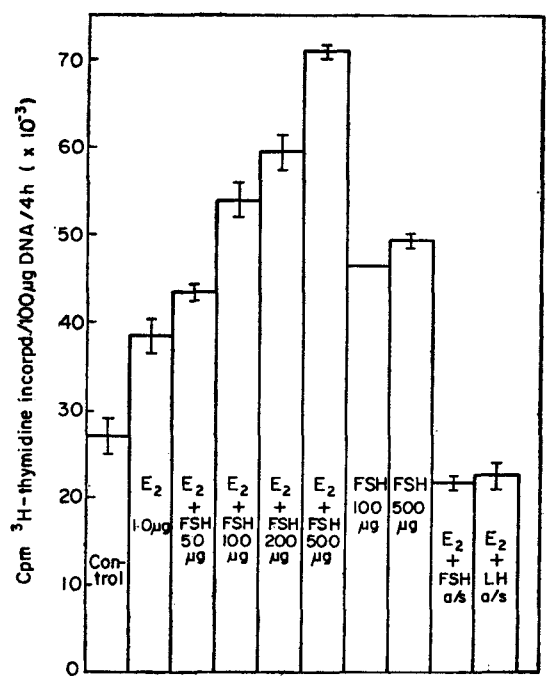

Figure 4. Effect of administration of E_2 (1·0 µg) in 0·9% NaCl, with or without various doses of FSH (50-500 µg NIH-FSH-S11) to 25 day-old immature rats. Other details as in legend to figure 3.

received along with saline or DES or E_2, different doses of FSH (NIH-FSH-S11) ranging from 50-500 μg, the two being given at separate sites on the back of the neck; yet another group of animals was given along with DES or E_2, specific antisera to FSH (25 μl) or LH (0·1 ml) intraperitoneally.

Administration of DES or E_2 to intact immature rats caused 18 hr later a significant increase ($p<0·001$) in the rate of *in vitro* incorporation of ^{3}H-thymidine into ovarian DNA (figures 3 and 4). Administration of increasing doses of FSH along with a constant dose of either DES or estradiol brought about a progressive increase in the response in a dose-dependent manner. The highest dose of FSH (500 μg) given along with estrogen caused a 2-fold increase over that seen with either DES or E_2. However, the same dose of FSH given alone without estrogen caused only a marginal increase over the control, the response being significantly less than that seen with estrogen +FSH. Further, as shown in figure 5, the combined response produced by the latter treatment was not simply additive, but synergistic i.e. greater than the sum of response to either estrogen or FSH treatment separately.

It was interesting to find that in the presence of antiserum to FSH, or LH, the response to either DES or E_2 was completely abolished (figures 3 and 4); similar response was observed when both estrogen and FSH were given along with LH antiserum. These experiments indicated that the presence of both the endogenous gonadotropins were necessary for stimulating of ^{3}H-thymidine incorporation into ovarian DNA by exogenous estrogen.

Results of the measurement of serum FSH and ovarian tissue bound FSH in untreated and estrogen-treated (for 24 hr) immature rats are shown in table 1. Treatment with DES or estradiol resulted in a significant increase in ovarian FSH concentration

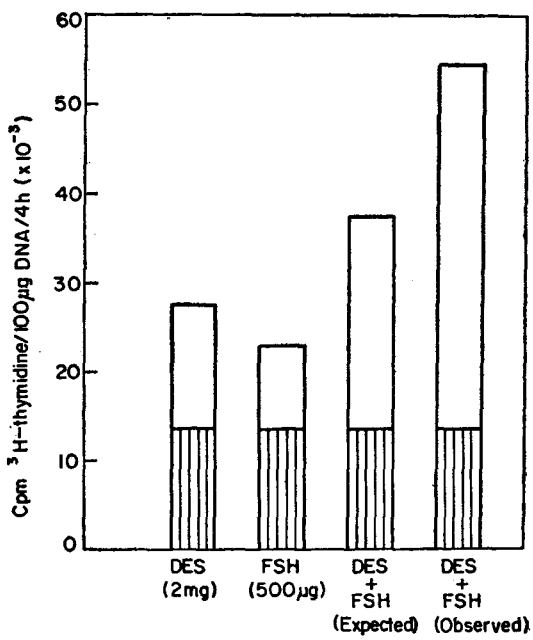

Figure 5. The expected and the observed pattern of response in terms of ^{3}H-thymidine incorporation into DNA *in vitro* of immature rat ovaries, to combined treatment with DES (2 mg) and FSH (500 μg). The shaded area represents the control value.

Table 1. Effect of treatment with DES or E_2 on ovarian tissue-bound and serum FSH concentration in immature rats

Treatment	FSH in ovary ng/10mg	FSH in serum ng/ml
Untreated or saline treated control	4·98± 1·00	146·2±62·77
DES (2 mg)	16·15± 0·99*	100·0±00·00
DES + FSH	14·70±00·07*	725·0±25·00†
E_2 (1 μg)	25·56±13·95*	270·0±72·70
E_2 + FSH	10·63±04·43†	525·0±23·63†

25 day-old immature female rats (N=5) were given subcutaneous injection of estrogen or estrogen +FSH (NIH-S11 500 μg), both in 0·1 ml of 0·9% NaCl at separate sites. Animals were killed 24 hr later and the ovarian and serum FSH levels measured by RIA as described under methods.

*These values are significantly different from the control at $p>0·001$

†These values are significantly different from the control at $p>0·01$.

by 24 hr. At this time, however, the serum FSH concentration in these treated groups did not show any significant increase over the control level. Surprisingly, even when the serum level of FSH was substantially increased by administering 500 μg FSH along with DES or E_2, there was no further enhancement in the concentration of ovarian FSH. In fact, in the group injected with estradiol and FSH, the tissue FSH concentration although higher than the control value ($p<0·01$), was significantly less than the concentration of FSH in the ovary of animals given E_2 alone.

4. Discussion

In the present study, we have investigated the induction of follicular growth in the ovary of immature rats and hamsters by two different hormonal stimuli—one, a gonadotropin and the other, a steroid. An early biochemical event that marks the initiation of follicular maturation in the ovary is DNA synthesis followed by cell multiplication; this has been monitored in the present study by determining the rate of incorporation of ^{3}H-thymidine into DNA of ovaries *in vitro*.

The time-course of stimulation of this activity in the ovary of immature hamster by PMSG was similar to that stimulated by endogenous gonadotropins in the ovary of adult cycling hamster (Sheela Rani and Moudgal 1977a). However, the duration for which the gonadotropin support was necessary to maximally stimulate the incorporation at 18 hr in these two systems appeared to be different, the immature animal needing it for a longer time (10–12 hr) than the adult (3–4 hr). Although neutralization of gonadotropin activity after this initial period of requirement did not affect this parameter measured 18 hr after the stimulus, it still could have blocked the overall process of follicular maturation in these animals. This has, in fact, been observed by us in the cycling hamster where neutralization of the second FSH surge by injecting FSH antiserum at 20.00 hr of proestrus, did not affect the rate of ^{3}H-thymidine incorporation into DNA of non-luteal ovarian tissue *in vitro* at 12.00 hr

of estrus, but did inhibit follicular growth during that cycle (Sheela Rani and Moudgal 1977a). Sasamoto and Kennan (1973) using ovulability as the parameter for follicular growth observed that in immature rats PMSG was needed for the first 36 hr and thereafter the endogenous gonadotropins were responsible for further development and maintenance of follicles in an ovulable state.

A change in morphological appearance of the ovary such as size and weight could be found only 24 hr after PMSG by which time the rate of ^{3}H-thymidine incorporation into DNA *in vitro* had declined. The wet weight of the ovary showed a steady increase up to 42 hr, which could perhaps be due to hypertrophy with little hyperplasia. During this period, in accordance with increased follicular growth, an increase in serum estradiol level was also found and this could be correlated to the increase in uterine weight, an observation similar to that of Parker *et al* (1976) in immature rats after PMSG administration.

Estrogen has repeatedly been demonstrated to act in hypophysectomized animals causing an enhancement of ovarian weight, cell-proliferation and increase in the number of receptors for gonadotropins (Fevold *et al* 1931; Williams 1940; Pencharz 1940; Simpson *et al* 1941; Goldenberg *et al* 1972; Richards *et al* 1976; Louvet and Vaitukaitis 1976). In the present study with intact immature rats, estrogen has been shown to similarly induce a response both in terms of increased ovarian FSH concentration and increased rate of ^{3}H-thymidine incorporation into ovarian DNA *in vitro*. However, our attempts to simulate a hypophysectomized condition (as far as the deprival of endogenous gonadotropins is concerned) using antisera to either of the gonadotropins yielded conflicting results. Since the effect of estrogen treatment on *in vitro* incorporation of thymidine into ovarian DNA could be completely inhibited by the simultaneous administration of antisera to either FSH or LH, it could be inferred that estrogen could be acting only via the endogenous gonadotropin(s). Further, a higher amount of endogenous FSH could be measured in the ovary of animals treated with either DES or E_2 compared to saline treated controls. In the same experiment, the reason for not observing an increase in serum FSH level could be that the time-lag between treatment and sampling (at autopsy) was quite long (24 hr). It is thus possible that estrogen could have caused an increased release of FSH at some earlier time period (which was not examined in the present case) and this might have returned to control levels by 24 hr. At this time, the fact that the tissue had higher concentration of FSH could perhaps signify a higher capacity of the tissue to retain the hormone by virtue of its increased receptor content.

Administration of a high dose of ovine FSH (with a view to increase serum levels) along with estrogen, however, did not further enhance the already increased FSH levels in the ovary and in one case, this actually resulted in a reduction in the concentration of FSH in the ovary compared to that found following estrogen treatment alone (table 1). It is possible that the failure to further increase the ovarian FSH concentration by a combined treatment of estrogen and FSH could be due to the availability of only a limited number of receptor sites, these being saturated with endogenous FSH; alternatively there could be occurring with an increase in dosage of FSH, a transient increase in tissue levels of FSH; this perhaps is not detected due to improper timing of measurement. In support of this is the observation that a combined treatment of a constant dose of estrogen with varying doses of FSH produced a progressive FSH dose-dependent increase in response in terms of ^{3}H-thymidine incorporation into DNA *in vitro* and this effect was clearly synergistic rather than additive (figure 5).

In addition, this system appears to have the potentiality of being developed into a sensitive bioassay for FSH. Since the purest ovine FSH preparation available is over 45–50 times as active as the NIH preparation used in the present study, the range used here corresponds to 1-10 μg of purified FSH; also a greater sensitivity perhaps could be achieved by working out the proper dose of estrogen.

In the light of the present observation that estrogen is unable to enhance *in vitro* incorporation of ^{3}H-thymidine into DNA in the absence of either endogenous FSH or LH, it is difficult to conceive how estrogen given alone to hypophysectomized rats does stimulate cell proliferation. An explanation to this controversial situation could be that even after hypophysectomy, some low undetectable amounts of gonadotropins are still present in the tissue (due to increased tissue half-life?) or that in the absence of trophic hormones of the pituitary the sensitivity of the tissue and its response to estrogen changes.

The present study thus points to a complex interplay of estrogen, FSH and LH in stimulating cell multiplication of the ovarian follicle in the intact animal. The mechanism by which such an interaction causes an enhancement in DNA synthesis, however, remains unknown.

Acknowledgements

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