THE RESPONSE OF THE IMMATURE RAT OVARY TO GONADOTROPHINS: ACUTE CHANGES IN CYCLIC AMP, PROGESTERONE, TESTOSTERONE, ANDROSTENEDIONE AND OESTRADIOL AFTER TREATMENT WITH PMS OR FSH + LH

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ABSTRACT

Radioimmunoassays were used to measure changes in progesterone, testosterone, androstenedione, oestradiol, gonadotrophin and ovarian cyclic AMP in immature female rats during the first 24 h after exposure to slowly (PMS) or rapidly (FSH + LH) disappearing gonadotrophins. Cyclic AMP was increased 30 min after injection of either kind of gonadotrophin but it had returned to control level within 4 h. Serum and ovarian testosterone and androstenedione also increased to a peak at 30 min but decreased to base line by the 4th h. Multiple injections of FSH + LH maintained an elevated serum testosterone level but they had little effect upon the secretion of androstenedione. Serum and ovarian progesterone increased quickly after treatment with gonadotrophin. With PMS the peak in the serum was reached at 8 h, it remained high for 4 h and then fell precipitously between the 12th and 16th h. FSH + LH produced a prompt increase in serum progesterone but the level could be maintained only by repeated doses given every 4 h. Oestradiol was not increased in the serum or the ovary until 20 h after

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PMS. One or two doses of FSH + LH did not produce an increase in oestrogen but a transient increase was found with 3 doses; 4 doses kept an elevated level of oestradiol for 12 h. These results indicate that the aromatizing system of the immature rat ovary is relatively inactive and that continual stimulation by gonadotrophin for about 10–12 h is necessary to bring about increased function. In contrast, the mechanisms for the synthesis and secretion of progesterone and androgens are very active and can be immediately stimulated by exposure to gonadotrophins.

The effects of exogenous gonadotrophins on the immature rat ovary have received considerable attention (Ahrén & Kostyo 1963; VanDyke & Katzman 1968; Reel & Gorski 1968; Ahrén et al. 1974; Koch et al. 1974; Nilsson et al. 1974; Nilsson & Selstam 1975). Whereas many ovarian changes can be shown to occur within minutes to a few hours after exposure to gonadotrophins the timing of increased steroidogenesis is less clear. Reel & Gorski (1968) reported that oestrogen secretion, as evidenced by increased uterine weight, was initiated within the first few hours after injection of PMS. Sashida & Johnson (1975) found no significant changes in serum oestradiol until 16 h after an injection of PMS. Wilson et al. (1974) found no change in either progesterone or oestradiol until about 28 h after PMS but they did not use frequent samplings during the first 24 h. The transient increase in progestins that has been reported for rabbits stimulated with gonadotrophins (Hilliard et al. 1968) suggested that possibly changes in the rat had been missed. The recent report of Kareim & Samuels (1974) added to this suspicion; they found steroid dehydrogenases were very active in the unstimulated immature mouse ovary but that aromatase was very inactive. If this condition prevailed in the rat we could expect a prompt increase in progesterone and androgen synthesis but a delay in oestrogen secretion. The present study was undertaken to delineate the temporal changes in these steroids in immature females treated with gonadotrophins. The use of PMS, which has a long half-life, enabled the study of responses without regard to continuation of gonadotrophin presence. The combination of FSH and LH was expected to give an equivalent qualitative stimulus but allow analysis of function under conditions of a rather rapidly declining gonadotrophin titer.

**MATERIAL AND METHODS**

Holtzman strain female rats (23–24 days old) were housed 10–12/cage and allowed free access to food and tap water. The room lights were on from 06.00–20.00 h daily and the room temperature was controlled at 23 ± 2°C. PMS (Ayerst Pharm. Co. or NIAMD) FSH (NIH-FSH-S-9) or LH (NIH-LH-S-15) were dissolved in 0.9 % saline; the dose to be administered was contained in 0.2 ml. [1,2,6,7-3H]Progesterone (105 Ci/mM); [1,2,6,7-3H]testosterone (85 Ci/mM); [1,2-3H]
Ovarian cyclic AMP content (pm/mg wet weight of ovary) at various times after an iv injection of 20 IU PMS at time 0. Control animals (1 and 16 h) were injected with saline. Each group has at least 5 animals and the SEM is shown by a vertical line.

Fig. 1.

Ovarian cyclic AMP content (pm/mg wet weight of ovary) at various times after an iv injection of 20 IU PMS at time 0. Control animals (1 and 16 h) were injected with saline. Each group has at least 5 animals and the SEM is shown by a vertical line.

androst-4-ene-3,17-dione (48 Ci/mM) and [2,4,6,7-3H]oestradiol-17β (90 Ci/mM) were obtained from New England Nuclear Corp. (Boston) Mass., Steroids used for standards were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Steroids were measured by radioimmunoassay using a specific antiserum for each hormone. Anti-oestradiol-17β was prepared and characterized by Exley et al. (1971). Serum concentrations below 5 pg/ml were not statistically different from serum blanks. Anti-progesterone-6-BSA and anti-testosterone-6-BSA were prepared in this laboratory; their characteristics in assays have been reported (Pang & Johnson 1974). Anti-progesterone could distinguish 5 pg/ml and anti-testosterone 10 pg/ml using water as a blank. Anti-androstenedione was a gift of Dr. H. R. Lindner (Weizman Institute, Rehovot, Israel): The reported specificity (Weinstein et al. 1972) was confirmed and when used at a final dilution of 1:500 it could detect 8 pg/ml compared to water as a blank.

Freshly opened anaesthetic grade diethyl ether was used for extraction of serum. For oestradiol 0.5 ml of serum was extracted once with 6 volumes of ether; oestradiol standards were extracted from 0.5 ml serum from adrenalectomized-ovariectomized adult female rats. For progesterone 10 μl and for the androgens 100 μl of serum was added to 0.5 ml deionized distilled water and then extracted with 3 ml ether. Internal standards were not used but extraction efficiency was determined using labelled steroids added to serum from adrenalectomized + ovariectomized female rats: the efficiency was 87.3 ± 1.3 %.

Steroid synthesis by ovaries exposed to PMS was evaluated by a 1 h incubation in Krebs-Ringer bicarbonate buffer (5 mM glucose). After weighing on a torsion balance the ovaries were placed in a 10 ml Erlenmeyer flask, gassed with 95% O₂ and 5% CO₂, sealed and placed in a shaking water bath at 37°C. The steroids were extracted from the ovaries by homogenization in methanol; blanks consisted of extracts of homogenized salivary glands. The incubation medium was extracted with 6 volumes of ether using the buffer as a blank.

Cyclic AMP was assayed by immunoassay using kits purchased from Collaborative Research Inc. (Waltham, Mass.). Ovaries were weighed and frozen in 6% trichlo-
Timing of changes in progesterone concentrations in immature rats given PMS. Tissue content was extracted after incubation of 2 ovaries for 1 h; the hormone released into the medium is shown in the centre. Circles (●) not connected indicate values for saline injected controls. Crosses (×) indicate hormone in animals adrenalectomized 72 h before the injection of PMS. 5 rats/group, SEM shown by vertical line if it exceeds the area covered by the symbol.

acetic acid. They were thawed, homogenized and centrifuged at 1200 × g for 30 min; the supernatant was washed twice with two volumes of acidified ether and then taken to dryness in a water bath. The residue was re-dissolved in 0.05 M sodium acetate (pH 6.2) for assay. The amount of cAMP in the samples was extrapolated from a standard curve over a range of 0.01 to 25 pm and expressed as pm/mg ovarian wet weight.

Serum gonadotrophin was measured by immunoassay. Rat and ovine LH (NIAMDD) were iodinated with 125I using a modification of the method of Butt (1972); the essential change was substitution of a small plastic paraffin-filled cup for the iodination vessel. Anti-rat LH (NIAMDD) or anti-ovine LHβ subunit (Dr. B. Goldman, Univ. Conn.) were used at a dilution which bound 35% of the label. Rat LH (RP-1) was used for a standard and the equivalent amount of LH in the serum was extrapolated from log-dose logit response curves. Statistical evaluations were done using Duncan's new multiple range test.
RESULTS

Injection of PMS

Animals were given 20 IU of PMS intravenously under light ether anesthesia and killed at various times thereafter. The amount of gonadotrophin in the blood was evaluated by use of an antiserum to the β subunit of ovine LH; a linear log-dose vs per cent bound curve over a range of 2 to 64 ng of LH-RH-1 was obtained. The first measurement at 30 min after the injection showed a serum LH value of about 1000 ng/ml (measured as LH-RP-1); after 12 h the level had decreased to 600 ng/ml. There was a decline to 200 ng/ml between the 12th and 16th h but it remained above this level through h 24. This confirms the expected presence of gonadotrophin over the period of the study.

Ovarian cyclic AMP increased promptly after PMS (Fig. 1) but it fell to the pre-injection level, or to that found in the controls given saline, within 4 h; no further changes were found h 16.

Steroid changes in the sera and the ovaries are shown in Figs. 2-5. The
large and rapid increase in serum progesterone was partially due to adrenal secretion as indicated by the level seen with saline injection and the reduced response obtained with adrenalectomized animals (Fig. 2). Peak levels were reached by h 8 in the serum with a large decline between h 12 and h 16. The amount of progesterone in the ovary, incubated for 1 h, or released into the incubation medium, reached a maximum at h 4 and then declined through h 16.

Testosterone (Fig. 3) and androstenedione (Fig. 4) rose quickly in the blood and ovary but also rapidly decreased and by h 4 both were about at the level found in controls. Serum testosterone showed a secondary rise in the serum and ovary beginning at about h 12. Androstenedione did not change in the serum after h 4; the ovarian content (medium not assayed) of controls given saline was higher at h 24 than in those treated with PMS.

Serum oestradiol increased significantly at h 20 (Fig. 5). The amount of oestrogen in the incubation medium, but not the ovary, was significantly higher than for controls during the first 2 h but between h 2 and h 12 there were no differences. After h 12 changes in serum, ovary and incubation medium were parallel.

Injection of FSH + LH

The effects of a single or multiple injections of 200 µg FSH and 20 µg LH were examined. The first injection was intravenous but subsequent doses were given subcutaneously. Results are shown in Figs. 6 and 7. Serum LH was still
very high at 30 min but it fell quickly. With multiple doses an LH level above 500 ng/ml was maintained for at least 4 h after each injection. At h 24 LH in controls was 15.2 ± 1.8 ng/ml whereas it was 49.3 ± 13 ng/ml in animals given 2 doses of hormone; 3 doses gave a value of 88.7 ± 24 ng/ml and 4 doses 116.7 ± 17 ng/ml. Animals given 4 doses had LH levels that remained above 225 ng/ml for at least 16 h.

The pattern of changes in cAMP was almost identical to that found with PMS (Fig. 6). Measurements were not made 30 min after each of the subsequent doses but only at the time of the next injection and at h 24 in all animals given 2 or more doses; cAMP was significantly higher ($P < 0.05$) at h 24 in animals given 3 or 4 doses of gonadotrophin but not 2 doses, when compared to those treated with saline.

The initial rise in serum progesterone (Fig. 7) no doubt is partly due to adrenal stimulation. The peak, about 20 ng/ml, is similar to that produced

Fig. 5.
Oestradiol in the serum, incubated ovaries and incubation medium at various times after injection of PMS. Symbols as in Fig. 2.
by PMS but with a single dose of FSH + LH the level returned to that of controls by h 4. A dose every 4 h kept the level up for 4 h but it always dropped during the succeeding 4 h. The rate of decline between the 4th and 8th h after the 4th dose was greater than that with 2 or 3 doses. At h 24 serum progesterone was not significantly different in animals treated with FSH + LH than in those injected with 1 or 4 doses of saline.

Increases in serum testosterone and androstenedione were transient with a single dose of FSH + LH; the peak at 30 min was quantitatively the same as with PMS (Fig. 7). Multiple doses kept serum testosterone elevated somewhat but they had little effect upon androstenedione. At h 24 the concentrations of both androgens were the same as in controls given saline.

Serum oestradiol (Fig. 6) did not become detectable in the serum of animals given 1 or 2 doses of FSH + LH. Three doses produced a transient increase and 4 doses kept the level up through h 24.

**DISCUSSION**

The present study has demonstrated that the immature rat ovary responds to gonadotrophic stimulation by a prompt increase in progesterone and androgen secretion. In contrast, oestrogen synthesis was not evident until many hours later. Thus it appears that the aromatizing system of the immature rat is relatively inactive as it is in the immature mouse (Kareim & Samuels 1974). The possibility of a “release” of ovarian oestradiol immediately after treatment with PMS was suggested by the *in vitro* incubation study. Whether this occurs *in vivo* is unknown but it could be responsible for initiating the increase in uterine weight that has been noted between 12 and 18 h after an injection of PMS (Reel & Gorski 1968); the increase in synthesis begins too late to account for this early uterine response.

A second important point is the requirement for continual gonadotrophic stimulation in order to obtain the ovarian oestrogen response. In our previous study (Sashida & Johnson 1975) we found that anti-PMS prevented the effect of PMS on oestrogen production even if it was given after oestrogen secretion had begun. Rapidly disappearing gonadotrophins further emphasize the need

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**Fig. 6.**

The effects of a single (left half) injection (iv) of 200 µg FSH and 20 µg LH in immature female rats. Results obtained with multiple injections are shown on the right half. Arrows indicate times of subcutaneous injections; assays were done at 4 h intervals after supplementary doses. Each group has at least 5 rats and SEM are indicated by a vertical lines when they exceed the area covered by the symbols. Open squares (□) indicated levels in controls given saline. Arrows on the graphs indicate values below the sensitivity of the assay.
Fig. 7.
Serum steroid changes with FSH + LH treatment. Same symbols as in Fig. 6.
for continual presence. With 2 doses of FSH + LH the serum gonadotrophin level would have remained elevated for at least 8 h and yet a rise in serum estradiol was not detected. With 3 doses plasma LH was up for at least 12 h and this caused a transient increase in oestradiol. Four doses raised LH for 16 h and serum oestradiol was elevated for at least 12 h. It is important to point out however, that even with the 4 doses the oestrogen concentration was going down between h 16 and h 24. In contrast, oestrogen increased exponentially after h 20 in animals given PMS. This rapid decay in gonadotrophic effect suggests that LH is the important hormone for steroidogenesis. Serum FSH concentrations were not measured but considering that ten times more FSH than LH was given at each injection and that it has a disappearance rate only about one-fifth that of LH (Gay & Sheth 1972) one would except very high concentrations in animals getting multiple doses. However, several studies (reviewed by Kareim & Samuels 1974) have shown that FSH has little effect upon ovarian steroidogenic enzymes.

The need for gonadotrophin in the oestrogenic response suggests that one function of the hormone is related to production of a material, or materials, which disappears quite rapidly. Lieberman et al. (1975), using an in vitro system involving ovarian follicles from mature cycling rats, suggested that a short-lived protein was essential for the effect of LH on steroidogenesis. Furthermore they proposed production of this protein was dependent upon a process sensitive to actinomycin D inhibition (mRNA?). It is uncertain whether the same processes are required for the immature ovary which has not been previously stimulated by gonadotrophins. Preliminary studies (Sashida & Johnson, unpublished) indicate some similarities however. Actinomycin D (200 μg) given 12 h after the PMS did not prevent the appearance of oestradiol at 20 h but by 24 h only 2 of 5 animals had detectable oestrogen. Cycloheximide (4 mg), an inhibitor of protein synthesis, completely prevented the rise in oestradiol at 20 h when it was administered at 12 h; delay of treatment until 16 or 20 h was progressively less effective in reducing the ability of the ovary to produce oestrogen. Combined with the results obtained with multiple injections of FSH + LH, this suggests that immature rat ovaries require about 12 h of gonadotrophic stimulation to begin to synthesize the essential protein (aromatase?) to bring about increased oestadiol synthesis.

Jarlstedt et al. (1973) have shown distinct qualitative changes in the metabolism of RNA in ovaries treated with gonadotrophin or with cAMP. The concept of cAMP as a "second messenger" has received a great deal of attention and its association with gonadotrophic action on ovaries has been discussed by Ahrén et al. (1974) and Nilsson et al. (1974). While there is no doubt that ovarian cAMP increases rapidly in response to many gonadotrophins we do not know whether it is essential for steroid production; it is certainly not adequate for the oestrogenic response as measured in the present
study. A single dose of FSH + LH produced the same change in cAMP as did the PMS but the former did not result in an increase in oestrogen. In contrast the oestrogen synthesizing system can be induced with low levels of endogenous gonadotrophins produced by repeated injections of exogenous LH-releasing hormone without causing any appreciable changes in ovarian cAMP (Sashida & Johnson, unpublished). Caution is necessary however, in interpreting such results because Ahrén et al. (1974) have shown that changes in cAMP can occur very rapidly and thus can be easily missed. Furthermore large changes in cAMP may not be necessary for a complete biologic effect and adequate but very transient changes could go undetected.

These studies have relied upon changes in immunoreactive hormones. In general the antisera were selected for their lack of cross-reactivity with known contaminants in serum and tissue but of course none have absolute specificity. Therefore absolute values have much less meaning than do the relative changes in concentrations. The results do allow us to conclude that the secretion of oestradiol, which is perhaps the most potent steroid of the ovary, requires a considerable period of stimulation by gonadotrophin. The amount of trophin necessary to accomplish the induction has not been determined but preliminary, unpublished, studies have indicated that it occurs with doses much lower than those used in the present study.

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