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STEROID PRODUCTION BY THE ISOLATED RABBIT OVARIAN FOLLICLE

I. EFFECTS OF OVINE LH

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Summary. Graafian follicles from mature New Zealand white rabbits were incubated for 4 hr at 37°C with air as the gas phase. The medium was changed every 15 min for 1 hr. Medium containing ovine LH was then added for four consecutive medium changes. Samples of the medium were analysed for 17β-hydroxyandrogens, oestrogens and progestins by radioimmunoassay procedures. When no LH was present, progestin and 17β-hydroxyandrogen secretion declined to negligible levels within 2 hr. Addition of 10 μg ovine LH to the medium for 1 hr caused an increase in 17β-hydroxyandrogen secretion which continued for the duration of the experiment. No significant change was observed in oestrogen secretion but there was a slight increase in progestin secretion. Addition of 5 μg and 1 μg LH for 15 min also caused an elevation of 17β-hydroxyandrogen secretion within 1 hr whereas progestin secretion took longer to increase and no change in oestrogen secretion was observed. No effect was observed with LH concentrations of less than 500 pg/ml for 1 hr, but 1 and 10 ng LH/ml also caused an increase in 17β-hydroxyandrogen secretion. Oestrogen secretion was also stimulated at these levels. Control incubations with interstitial tissue showed no oestrogen secretion, slight secretion of 17β-hydroxyandrogens and greater secretion of progestin which was stimulated by exogenous LH.

INTRODUCTION

Evidence that the mammalian ovary secretes androgens was first provided by Hill (1937) who showed that ovarian grafts can restore seminal vesicle and prostate glands of castrated male mice. In the human polycystic ovary syndrome when androgens are secreted in large amounts (Abraham, Marshall & Daane, 1972), it is believed that the ovarian stroma provides the source of these androgens (Savard, Marsh & Rice, 1965). The interstitial tissue of rabbit ovaries has an endocrine rôle in facilitating gonadotrophin release through 20α-hydroxypregn-4-en-3-one (Hilliard, Spies & Sawyer, 1969). Rabbit ovaries can produce androgens *in vivo* (Hilliard, Scaramuzzi & Pang, 1973; YoungLai, 1973a) as well as *in vitro* (Gospodarowicz, 1964; Mills & Savard, 1972, 1973; Coulson, Liu, Morris & Gorski, 1972). In the latter studies, radioactive precursors were used to examine steroidogenesis.

The present investigation was undertaken to determine testosterone (17β-hydroxyandrogen) production by the isolated rabbit follicle and the effects of exogenous LH on this production. Oestrogen and progestin secretion were also examined at the same time to ascertain whether changes in androgen secretion are accompanied by changes in oestrogen and progestin secretion.

MATERIALS AND METHODS

Unless otherwise stated, the materials and methods were the same as those previously described (YoungLai, 1972a). Mature New Zealand white rabbits were anaesthetized and their ovaries were removed. Usually, two to three animals were used to provide follicles which were then randomly allotted to groups of five to twelve. The follicles were slit and incubated in 1-ml portions of medium consisting of Hanks balanced salt solution (BSS): Medium 199: normal rabbit serum (55:30:15, by vol.). The follicles were placed in the centre wells of 60 × 15 mm organ tissue culture dishes (Falcon Plastics) with the outer absorbent ring soaked in BSS. The medium was replaced every 15 min and the withdrawn samples were stored at −15° C until assayed. Incubations were performed at 37°C in a National CO_2 incubator, with air as the gas phase. In some experiments, interstitial tissue was substituted for follicles. Ovine LH (NIH-LH-S-16) was added to the incubation media during the following procedures: (i) 10 μg LH/ml at medium changes 5 to 8, i.e. 1-hr exposure to LH—four experiments with follicles; (ii) 500 ng LH/ml as in (i)—two experiments; (iii) 10 ng LH/ml as in (i)—one experiment; (iv) 1 ng LH/ml as in (i)—one experiment; (v) 250 pg LH/ml as (i)—one experiment; (vi) 500 pg LH/ml as in (i) but follicles were kept at 4°C overnight and then stimulated for 1 hr with 5 μg LH—one experiment; (vii) 5 μg LH/ml at medium change 5, i.e. 15-min exposure of follicles to LH—one experiment; (viii) 1 μg LH/ml as in (vii)—one experiment; (ix) same conditions as in (i)—two experiments with interstitial tissue.

Duplicate control experiments without any added LH were performed with follicles as well as interstitial tissue. Supplies of [6,7-^{3}H]oestradiol-17β (48 Ci/mmol), [1,2-^{3}H]testosterone, (45 Ci/mmol) and [1,2-^{3}H]progesterone (50·3 Ci/mmol were purchased from the New England Nuclear Corporation and were checked for purity by paper chromatography before use. Radioactivity was measured in a Beckman LS200 liquid scintillation spectrometer.

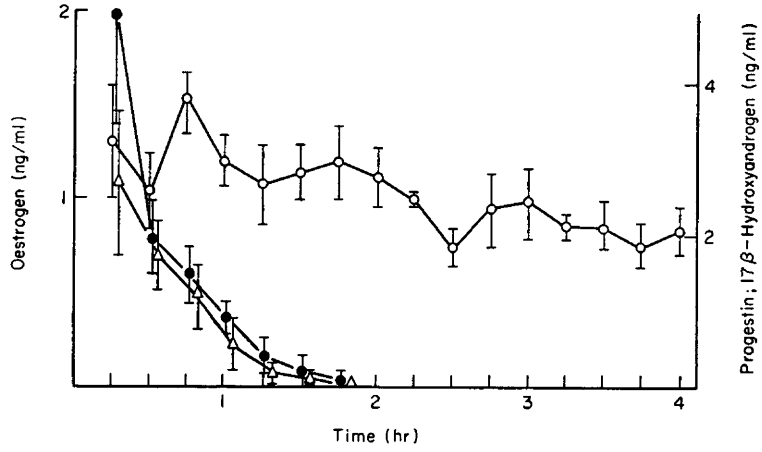
Radioimmunoassays were carried out as outlined previously (YoungLai, 1972a, 1973a). No chromatography step was included in the assays. Thus, for the oestrogen assay, the method probably measured only oestrone and oestradiol which are the oestrogens made and secreted by the rabbit follicle (Mills, Davies & Savard, 1971). Since the testosterone antiserum cross-reacts with 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), the results have been expressed as 17β-hydroxyandrogens although we have not been able to isolate dihydrotestosterone in incubations of follicles with labelled testosterone and androstenedione (YoungLai, 1972a). The progesterone antiserum cross-reacts with 17α-hydroxyprogesterone and other adrenal corticoids but not to any great extent with 20α-hydroxypregn-4-en-3-one (Abraham, Swerdloff, Tul-

chinsky & Odell, 1971). Media were diluted 1:5 in Buffer A and different aliquots were taken for direct assay. The results represent total progestins, the bulk of which consists of progesterone and 17α-hydroxyprogesterone. In all assays, immunoreactivity of medium alone was substracted from the results. These values were very low even when medium alone was incubated for up to 8 hr.

Statistical analysis was carried out as outlined by Snedecor & Cochran (1967).

RESULTS

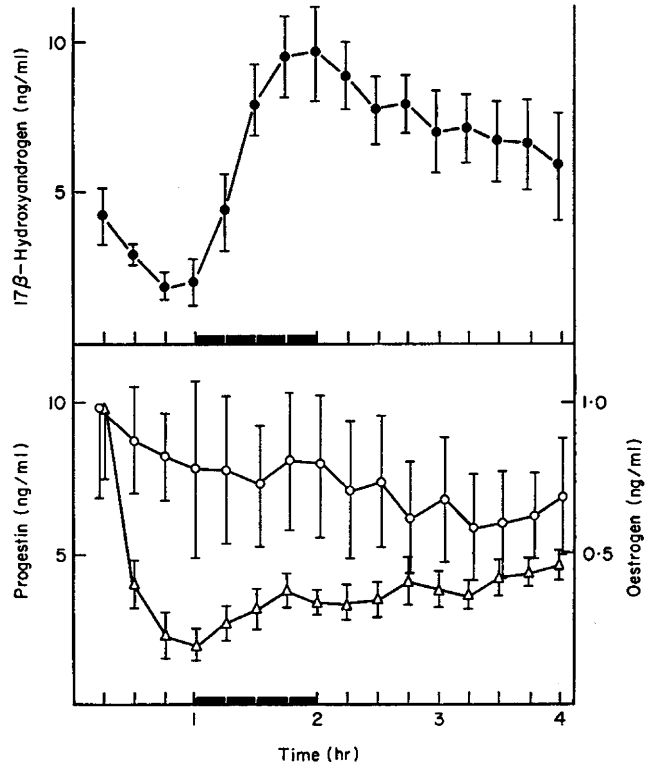
The secretion patterns of the three groups of steroids by isolated follicles without exogenous LH are shown in Text-fig. 1. The follicles, obtained from three rabbits with uteri weighing 9·80, 22·46 and 15·49 g, were incubated six to a dish



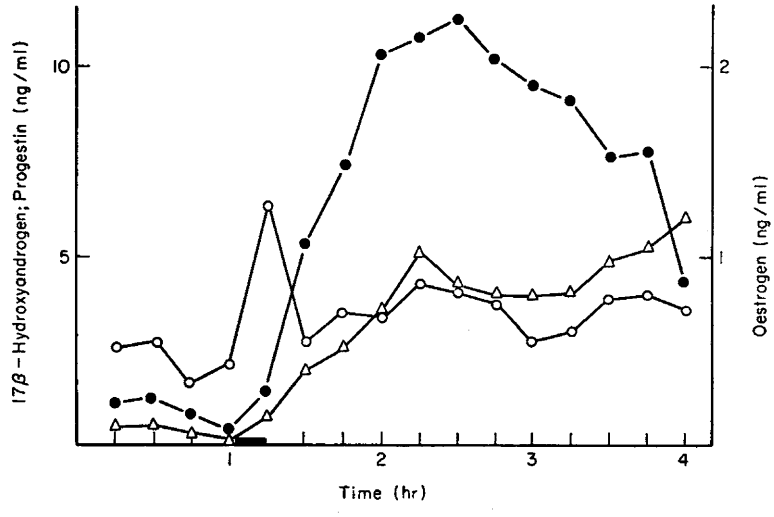
TEXT-FIG. 1. Steroid secretion by rabbit follicles. The results are expressed as the mean and standard error of two experiments. ●, 17β-Hydroxyandrogen; ○, oestrogen; △, progestin.

without LH. It is evident from Text-fig. 1 that secretion of progestins and 17β-hydroxyandrogens was negligible within 2 hr. Oestrogen values, however, showed a steady release which tended to decline at the end of the 4 hr.

When medium containing 10 μg LH/ml was added during the 2nd hr of incubation, there was a dramatic increase in secretion of 17β-hydroxyandrogen whereas there was no significant change in oestrogen and only a slight increase in progestin (Text-fig. 2). In two of the four experiments, eight follicles each were used (two rabbits with uterine weights of 7·89 and 7·36 g); in the other two experiments, the follicles from two rabbits (uterine weights of 21·85 and 13·61 g) were divided into three groups of five. The follicles in two of these latter groups were incubated with LH while those in the third were homogenized in 1 ml medium and analysed for steroids. Values for the homogenate were 3·9 ng 17β-hydroxyandrogens/ml, 1·3 ng oestrogens/ml and 24 ng progestin/ml. The



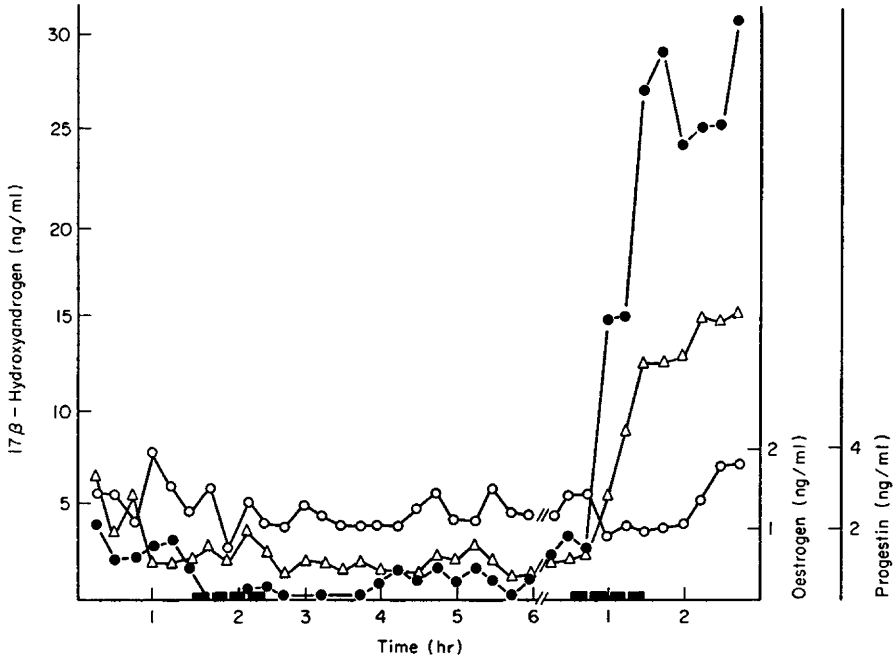
TEXT-FIG. 2. Effect on steroid secretion by rabbit follicles of exposure to 10 μg LH/ml for 1 hr. The results represent the mean and standard error of four experiments. ●, 17β-Hydroxyandrogen; ○, oestrogen; △, progestin. The bars represent the LH administration.



TEXT-FIG. 3. Effect on steroid secretion by rabbit follicles of exposure to 5 μg LH/ml for 15 min. ●, 17β-Hydroxyandrogen; ○, oestrogen; △, progestin. The bar represents the LH administration.

corresponding values for the homogenates at the end of all four experiments were 4·04±3·3 ng/ml, 1·04±0·76 ng/ml and 15·9±5·1 ng/ml, respectively.

When the dose of LH was reduced to 5 μg/ml and the duration of exposure decreased to 15 min, there was a marked increase in 17β-hydroxyandrogen secretion. Twelve follicles were used from two rabbits (uterine weights of 2·42 and 6·13 g). Text-figure 3 shows the pattern of steroid secretion from these follicles. Doses of 1 μg, 500 ng, 10 ng, and 1 ng (Procedures ii to iv inclusive, and viii) gave essentially the same responses as those shown in Text-fig. 3, except that there was a two- to threefold increase in oestrogen secretion.

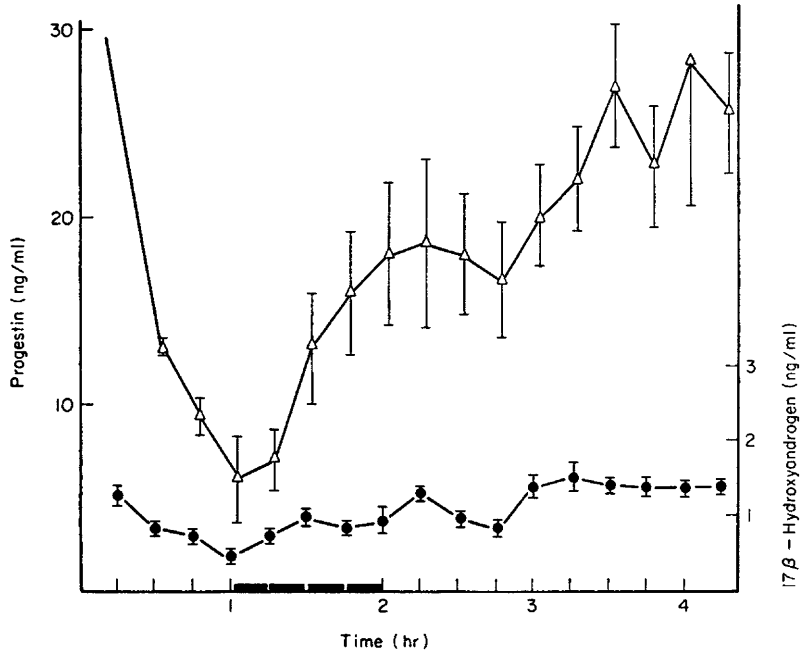


TEXT-FIG. 4. Effect on steroid secretion by rabbit follicles of exposure to 500 pg LH/ml for 1 hr and 5 μg LH/ml for 1 hr after storage at 4°C for 15 hr. Samples from 1½ to 2½ hr were collected after treatment with 500 pg LH/ml. The discontinuity of the graphs represents overnight storage at 4°C. Then, at ½ to 1½ hr, 5μg LH/ml was added at each medium change. ●, 17β-Hydroxyandrogen; ○, oestrogen; △, progestin. The bars indicate the time of exposure to LH.

Exposure of follicles to LH at levels of less than 500 pg (Procedures v and vi) for 1 hr failed to elicit any response. All steroid levels declined as shown by the data in Text-fig. 1. When the same follicles were incubated the next day, having been kept at 4°C overnight, a good response to 5 μg LH/ml for 1 hr was observed (Text-fig. 4). A total of nineteen follicles obtained from two rabbits with uteri weighing 6·54 and 6·71 g were used. Progestin and 17β-hydroxyandrogen secretion increased after LH stimulation but the response of the latter was much greater than that of the former.

When interstitial tissue from mature rabbits (the same as those used for the data of Text-fig. 3) was checked for the secretion of steroids, oestrogens and

17β-hydroxyandrogens were only detected in minute amounts in the first three samples. Progestin values were initially high and then decreased to low levels and remained low but detectable for the duration of the incubations. The homogenates at the end of the incubations had the equivalent of 7·2 and 5·6 ng/ml progestin. Oestrogens and 17β-hydroxyandrogens were not detectable. On addition of LH to interstitial tissue (two rabbits, uterine weights of 5·65 and 7·44 g), there was a gradual increase in progestin secretion within 1 hr followed by a larger increase in the 4th hour (Text-fig. 5). Tissue homogenates at the



TEXT-FIG. 5. Effect of LH on steroid secretion by rabbit interstitial tissue. The results are expressed as the mean and standard error of two experiments. ●, 17β-Hydroxyandrogen; △, progestin. The bar represents the period of exposure to LH.

end of the incubations had the equivalent of 16 and 40 ng/ml progestin. Oestrogens were not detected and androgens were close to the limit of sensitivity of our testosterone assay. Although there seemed to be some androgen secretion, the differences were not as dramatic as for the follicular incubations.

DISCUSSION

The follicles are generally regarded as the primary source of preovulatory oestrogen (Hisaw, 1947). Follicles may also contribute to preovulatory progestin secretion in the rat (Stoklosowa & Nalbandov, 1971), hamster (Leavitt, Bosley & Blaha, 1971) and rabbit (Hilliard & Eaton, 1971; YoungLai, 1972b). The rabbit follicle is able to synthesize testosterone from acetate (Mills & Savard, 1972) and has been implicated in testosterone secretion *in vivo* (Hilliard *et al.*, 1973; YoungLai, 1973a). The present investigation with isolated follicles

confirms and extends the observation that the rabbit follicles are better sources of androgens than of oestrogens and progestins when stimulated by LH alone.

Because of the well-known negative feedback rôle of androgen on the secretion of gonadotrophins, it is possible that follicular androgen serves to limit the amount of LH released by the pituitary. Recent evidence with androgen-insensitive mice suggests that androgens may be needed for normal ovarian function (Ohno, Christian & Attardi, 1973), but gross ovarian function in rats is not affected by androgens (Neumann, von Berswordt-Wallrabe, Elger, Steinbeck, Hahn & Kramer, 1970). Although it is known that steroidogenesis is related to meiotic maturation (Snyder & Schuetz, 1973), evidence obtained in rats indicates that oocyte maturation is not mediated by progesterone (Lindner, Tsafriri & Zor, 1974). The physiological rôle of androgens in the female rabbit warrants further investigation.

It is of interest that oestrogen is slowly released in a continuous manner for the duration of the experiment *in vitro* whereas progestin and 17β-hydroxyandrogen release ceases after 2 hr. This suggests that the follicles produce oestrogen continually whereas progestin and 17β-hydroxyandrogen production require constant stimulation. The last two groups of steroids probably represent material originally stored in the follicles which is released spontaneously. The continuous release of oestrogen and the fact that the follicles will respond to LH with an increased release of 17β-hydroxyandrogen after 15 hr in the cold (Text-fig. 4) suggest that the cells remained viable.

The precursors of 17β-hydroxyandrogen affected by stimulation with LH remain in doubt. If progesterone fulfilled this function one would have expected a fall in progestin values relative to the increase in 17β-hydroxyandrogen as demonstrated by Moor, Hay, McIntosh & Caldwell (1973) for progesterone and oestrogen in the sheep follicle. Gonadotrophins stimulate both synthesis and release of steroids (Eik-Nes, 1964). Differences in rate of release of progestin relative to androgen and the fact that the homogenates of follicles contained about sixfold more progestin before incubation could account for the observed effects. Moreover, it is known that LH has an inhibitory effect on the 17β-hydroxysteroid dehydrogenase for androstenedione (Boucek, Telegdy & Savard, 1967; YoungLai, 1973b). Thus, it seems unlikely that the Δ^4 pathway through progesterone would be activated for testosterone synthesis but it is possible that a steroid such as Δ^5-androstene-3β,17β-diol may be a logical precursor.

In view of the increasing evidence that steroids are released in a pulsatile fashion (West, Mahajan, Chavre, Nabors & Tyler, 1973), it is noteworthy that testosterone and progestin secretion by the follicles showed a gradual increase and slow decline over a 3-hr period following a 15-min stimulation with LH (Text-fig. 3). This observation argues against the view that episodic release originates in the gonad unless one considers that a pulse could be of 3 hr duration. With a similar preparation using pituitaries, Weisz & Lloyd (1972) showed that LH is released in a pulsatile manner when stimulated with median eminence extract. These authors also suggested that gonadal steroids are released episodically.

It is known that follicular cells have specific receptors for LH (Rajaniemi & Vanha-Perttula, 1972; Kammerman, Canfield, Kolena & Channing, 1972),

which have been utilized for the assay of LH (Shirley & Stephenson, 1973). It is only after the LH is bound that a cascade of events take place leading, among other things, to steroidogenesis. This could explain why androgen is not released in a pulsatile manner in the follicular incubations since pulse release probably represents secretion of stored material and, in these experiments, androgen secretion was minimal before the addition of LH. It is also possible that exposure to LH for 1 hr (Text-fig. 2) is more than ample and the only requirement is sufficient LH to saturate the binding sites on the follicular cells, as in the 15-min exposure which gave an identical response with respect to androgen secretion (Text-fig. 3).

The absence of stimulation of oestrogen secretion by 10 μg LH is difficult to explain. Mills *et al.* (1971) found that rabbit follicles were extremely sensitive to LH resulting in a large incorporation of [1-^{14}C]acetate into oestrogens. At lower levels of LH (Text-fig. 3), there was some stimulation of oestrogen secretion. Hilliard & Eaton (1971) demonstrated that oestrogen secretion *in vivo* is increased by mating, and previous data indicate that follicular fluid oestrogen is enhanced by mating (YoungLai, 1972b). On the other hand, LH can terminate oestrogen secretion and promote progestin secretion in the oestrous rat (Hori, Ide & Miyake, 1969). Thus, the high levels of LH used could be inhibitory for the aromatizing system.

Progestin secretion by interstitial tissue and its stimulation by LH is not novel (Major & Kilpatrick, 1972). The low level of androgen secretion is contrary to a similar rôle for interstitial tissue in the human ovary (Savard *et al.*, 1965), and also to the situation *in vivo* where the interstitial tissue of the rabbit is presumed to be involved in androgen secretion (Hilliard *et al.*, 1973).

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