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PRE- AND POST-OVULATORY CHANGES IN THE LEVELS OF STEROID HORMONES OF RABBIT OVARY ON *IN VIVO* ADMINISTRATION OF LH

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SUMMARY

Injections of LH (1 μg) directly into the stroma induced differential steroidogenic response in follicles and stroma of oestrous rabbit ovary. Stromal tissue responded to LH within 1 h with increased amounts of progesterone and testosterone in addition to high concentrations of 4-androstene-3, 17-dione and oestradiol acetate, which were already present at oestrus. At 6 h follicles contained peak quantities of progesterone, oestrogen acetates, 4-androstene-3, 17-dione and dehydroepiandrosterone. By 8 h all the hormone levels were low in both the tissues. A preovulatory second rise in progesterone and testosterone acetate of follicles was observed at 11 h. This rise was probably related to luteal changes in the follicles, since corpus luteum also contained higher amounts of these steroids. The steroidogenic response of follicles and stroma after intravenous injection of LH followed a similar pattern to that of the intraovarian one.

Corpora lutea contained greater amounts of steroid hormones than did the mature follicles. Of the steroid acetates, testosterone and oestradiol acetates were present in all three compartments of the LH stimulated ovary. The levels of oestradiol acetate in follicles and stroma could be correlated with the reported changes in oestradiol concentrations in thecal cells and ovarian vein blood of rabbits after coitus or LH injection. In the follicles and stroma of contralateral ovary, in which no ovulation occurred, the predominant steroids were oestrone acetate and androgens, unlike the changes observed in LH injected ovary.

INTRODUCTION

In rabbit, a reflex ovulator, copulation initiates the surge of luteinizing hormone (LH) followed by ovulation within 11–12 h [1]. Recent studies on the intrafollicular [2] and intraovarian [3] injections of LH in the rabbit further implicate a direct role for LH in the process of ovulation. Coitus or injection of LH or hCG brings about an increase in the levels of progestins and oestrogens [4–7] in the ovarian vein blood within 1–3 h. The contribution of either follicles or stroma towards this increased steroidogenesis has not been elucidated. In the present study, the effect of intraovarian and intravenous injection of LH on the levels of progesterone, pregnenolone, 4-androstene-3, 17-dione, dehydroepiandrosterone, testosterone and its acetate and phenolic steroids at different time intervals in follicles, stroma and corpora lutea of rabbit ovary has been investigated.

MATERIALS AND METHODS

The steroid hormones were purchased from the Sigma Chemical Co., or from Steraloids Inc. (U.S.A.) and checked for purity by thin-layer chromatography (t.l.c.) on silica gel G plates. The radioactive steroids, [4-^{14}C]-androstene-3,17-dione (S.A. 57.5 mCi/mmol), [4-^{14}C]-dehydroepiandrosterone (S.A. 52 mCi/mmol), [4-^{14}C]-progesterone (S.A. 61 mCi/mmol), [4-^{14}C]-pregnenolone (S.A. 51 mCi/mmol) and [4-^{14}C]-testosterone (S.A. 50 mCi/mmol) were supplied by the Radiochemical Centre, Amersham. Ovine LH (S-16) was a gift of National Institute of Child Health and Human Development, Bethesda, MD. All solvents were purified and distilled before use.

Virgin rabbits weighing about 2 kg were purchased from local suppliers and kept isolated under laboratory conditions for one month.

Intraovarian injections of LH on steroid hormone levels of follicles and stroma of treated and contralateral ovaries. Intraovarian injections of LH (1 μg) in saline or the vehicle alone were given as described earlier [8, 9]. At intervals of 1, 2, 4, 6, 8, 10 and 11 h after administration of LH, the rabbits were sacrificed. Ovaries from three individual rabbits were pooled together for each time interval and mature follicles and stroma of treated and contralateral ovaries were separately frozen at −15°C. The tissues were homogenized in 3 ml of 5 N sodium hydroxide solution after adding ^{14}C-labelled steroids (5000 d.p.m.) to each sample for correction of losses. The homogenates were extracted with ether (3 × 20 ml) and the solvent was evaporated to dryness. The residue was partitioned between light petroleum–benzene (1:1, v/v, 30 ml) and 1.6% sodium hydroxide solution (30 ml). The former fraction containing neutral steroids, was washed twice with water (25 ml) and dried under vacuum at 50°C. It was then chromatographed on silica gel G plates in chloroform–acetone (98:2, v/v) solvent system. The area corresponding to

testosterone acetate was eluted and rechromatographed in benzene–ethyl acetate (8:2, v/v) solvent system. The other areas corresponding to progesterone, 4-androstene-3,17-dione, pregnenolone, dehydroepiandrosterone and testosterone were eluted with chloroform–methanol (1:1, v/v). These were acetylated by adding pyridine (0.1 ml) and acetic anhydride (0.1 ml) and kept overnight in dark at room temperature (28°C). The compounds were dried under nitrogen at 50°C and rechromatographed using benzene–ethyl acetate solvent system in different proportions (8:2 and 9:1, v/v). The relative mobilities of neutral steroids in three different solvent systems are shown in Table 1.

The aqueous sodium hydroxide fraction, containing phenolic steroids, was methylated by adding boric acid (0.9 g) and dimethyl sulphate (1 ml). The solution was heated at 50°C and shaken vigorously until all dimethyl sulphate and boric acid dissolved. The procedure was repeated by adding more dimethyl sulphate (1 ml) together with 20% sodium hydroxide solution (2 ml). The oestrogens were extracted with benzene (2 × 25 ml) after allowing the flask to stand overnight at room temperature (25–28°C). The benzene extract was washed twice with water (30 ml) and then dried. The residue was chromatographed in cyclohexane–ethyl acetate (7:3, v/v) solvent system. Fractions corresponding to methyl ethers of oestrone and oestradiol-17β were eluted. The mobilities of free and esterified phenols in the above solvent system are presented in Table 2.

Gas–liquid chromatography. Progesterone, testosterone, dehydroepiandrosterone, pregenolone, 4-androstene-3,17-dione, testosterone acetate and methyl ethers of oestrogens were analysed on a Packard Gas-Chromatograph (Model 800 Series). Coiled columns of 1% XE-60 on Gas Chrom Q support with a hydrogen flame ionization detector were used. The flow of nitrogen was maintained at 60 ml/min and that of air and hydrogen at 300 and 30 ml/min respectively. The oven temperature was fixed at 220–230°C depending upon the retention times of hormones to be analysed. The relative mobilities of neutral and phenolic steroids are presented in Tables 1 and 2. The compounds were further checked on 3% SE-30, a non-selective phase for steroids.

Liquid scintillation counting. Aliquots of all hormones were evaporated to dryness and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3320) after adding 12 ml of scintillation fluid containing 3 g of PPO/l. of toluene.

Intravenous injections of LH on the hormone levels of follicles and stroma. In another group of rabbits, intravenous injections of LH (10 μg) in 0.2 ml saline solution were given through the ear vein. Follicles and stroma obtained at 1 and 4 h after LH injection were stored at −15° and processed for steroid hormones as described above. The phenolic steroid fraction was, however, treated differently. It was adjusted to pH 9.5 and extracted with ether (3 × 20 ml) and the ether extract was evaporated after washing with water. The dried fraction was chromatographed in cyclohexane–ethyl acetate (7:3, v/v) solvent system. The phenolic acetates were well separated from oestrone and oestradiol-17β (Table 2). The acetates were further purified by t.l.c. in benzene–ethyl acetate (8:2,

Table 1. Relative mobilities of neutral steroids on chromatography

Steroid hormone	Thin-layer chromatography R_T 1	2	3	g.l.c. on 1% XE-60 at 220° R_{rT}
Cholesterol	2.58	—	—	—
Progesterone	3.06	3.12	4.20	1.59
Pregnenediol	0.03	—	—	—
4-Androstene-3,17-dione	2.06	2.12	2.70	1.12
Androstenediol	0.78	—	—	—
Pregnenolone	1.93	—	—	—
Dehydroepiandrosterone	1.54	—	—	—
Testosterone	1.00	1.00	1.00	1.00
Dihydrotestosterone	1.77	—	—	—
20α-Dihydroprogesterone	1.03	3.36	3.70	1.55
17α-Hydroxyprogesterone	0.87	0.96	0.70	—
Testosterone acetate	3.45	3.12	3.60	1.08
Dehydroepiandrosterone acetate	solvent front	4.92	8.00	0.59
Pregnenolone acetate	solvent front	5.24	9.20	0.81
Cholesterol acetate	solvent front	6.08	14.20	1.02
Androstenediol diacetate	solvent front	5.08	9.70	—
Pregnendiol diacetate	solvent front	—	—	0.89
20β-Dihydroprogesterone	1.03	—	—	1.34

R_T = Ratio of mobility in relation to testosterone taken as unity. R_{rT} = Relative retention time with reference to testosterone taken as unity. Solvent systems: 1 = Chloroform–acetone (98:2, v/v); 2 = benzene–ethyl acetate (8:2, v/v); 3 = benzene–ethyl acetate (9:1, v/v).

Table 2. Relative mobilities of phenolic steroids on chromatography

Steroid hormone	T.L.C. in cyclohexane–ethyl acetate (7:3, v/v) $R_{E_1\text{–Meo}}$	G.L.C. on 1% XE-60 at 220° R_t
Oestrone	0.49	18.4
Oestradiol-17β	0.27	18.4
Oestradiol-17α	0.37	—
Oestrone-3-methyl ether	1.00	8.0
Oestradiol-3,17-methyl ether	0.55	7.6
Oestrone acetate	1.00	16.8
Oestradiol acetate	0.67	17.6
Oestriol acetate	0.56	—

$R_{E_1\text{–Meo}}$ = Relative mobility of the steroids with reference to oestrone-3-methyl ether taken as unity.
R_t = Retention time in minutes.

v/v) solvent system. The free oestrogens were acetylated and rechromatographed in the same solvent system. These were not detectable on g.l.c.

Steroid hormone levels in follicles, corpora lutea and stroma at different stages of ovarian function. Follicles and stroma were collected from oestrous rabbit ovaries. Corpora lutea were obtained from ovaries of rabbits after 48 h of intraovarian injections of LH. The tissues were processed for analysis of hormone levels as those obtained after intravenous injections of LH.

The method gave recoveries ranging from 36.4–74.3%, with pregnenolone showing low recovery (Table 3). This may be due to its sparing solubility in most of the solvents.

RESULTS

Effect of intraovarian injection of LH on steroid hormone levels in the treated and contralateral ovaries. The oestrogen methyl ethers, obtained after methylation of the phenolic steroid fraction extracted from the tissues, showed higher retention times on injection into g.l.c. columns than the corresponding standard methyl ethers of oestrone and oestradiol-17β. Labelled oestrone and oestradiol-17β added to the tissues for correction of losses were, however, recovered as methyl ethers in 50–60% yield. The peak heights of the unknown compounds varied with different time intervals of injection of LH in a definite pattern. The mobilities of these compounds on both t.l.c. and g.l.c. corresponded to oestrone and oestradiol acetates (Table 2). Injection into 3% SE-30 column gave retention times of 17.7 and 28.8 min for oestrone and oestradiol acetates respectively. The fractions, identified as oestradiol acetate, were pooled together and absorption in the ultraviolet light was measured. The compound showed maxima at 273, 268 and 230 mμ as standard oestradiol acetate. Enzymatic hydrolysis of oestrone and oestradiol acetates with acetyl cholinesterase following the procedure of Gandy and Peterson[10] resulted in free oestrone and oestradiol-17β as evidenced from their mobilities on t.l.c. and g.l.c.

(a) *Follicles.* The hormonal content of follicles of LH injected and contralateral ovaries at 1, 2, 4, 6, 8, 10 and 11 h after injection of LH is shown in Fig. 1. At 1 h of administration of LH, the follicles of the injected ovary contained more of testosterone acetate and 4-androstene-3, 17-dione compared to the other hormones. The levels of the latter along with testosterone showed increased concentration at 2 h. After 6 h, the amount of progesterone, oestrone acetate and oestradiol acetate reached peak levels with androgens being low. The levels of all hormones dropped to minimum by 8 h. While the other hormones still remained low, a second rise in progesterone and testosterone acetate was observed at 10 and 11 h.

Table 3. Recoveries of steroid hormones

Steroid hormone	No. of duplicates	Mean recovery %	Coefficient of variation %
Oestrone acetate	7	64.1	10.5
Oestradiol acetate	8	74.3	16.0
Testosterone acetate	8	41.3	22.0
Progesterone	10	54.6	7.8
4-Androstene-3, 17-dione	10	52.0	3.5
Pregnenolone	10	36.4	14.5
Dehydroepiandrosterone	10	42.3	9.4
Testosterone	10	57.6	6.8
Oestrone (as methyl ether)	6	47.5	8.4
Oestradiol (as methyl ether)	6	54.6	12.6

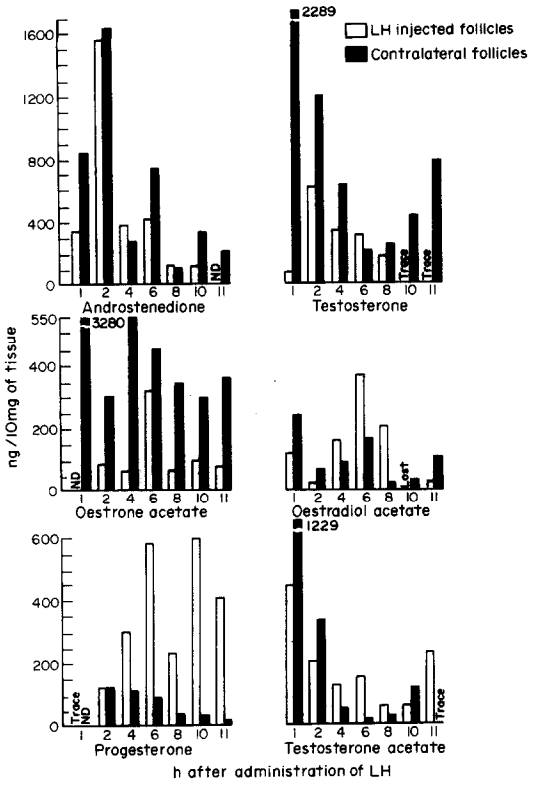

Fig. 1. Steroid hormone content of follicles at 1, 2, 4, 6, 8, 10 and 11 h after intraovarian injection of LH.

The contralateral follicles had high concentrations of oestrone acetate at all time intervals of analysis. At 1 and 2 h, testosterone acetate, testosterone and 4-androstene-3,17-dione were relatively more in the contralateral follicles than in the injected ones at the same time interval. Progesterone and oestradiol acetate were, however, not stimulated in a similar manner in these follicles as in the LH injected ones.

(b) *Stroma.* At 1 h after LH injection, the stroma contained progesterone, oestradiol acetate, 4-androstene-3,17-dione and testosterone in higher quantities than oestrone acetate and testosterone acetate (Fig. 2). All the hormones gradually dropped to low levels, while progesterone showed a second rise at 6 h. Testosterone, 4-androstene-3,17-dione and testosterone acetate showed elevated levels again at 11 h. The stroma of contralateral ovary had similar pattern of androgens at 1 h as in the injected ovary. Progesterone, oestrone acetate and oestradiol acetate were, however, low and showed increased levels at later time intervals. The levels of oestrone acetate continued to remain high even at 10 and 11 h in the stroma of the contralateral ovary. In addition, it contained detectable amounts of dehydroepiandrosterone and pregnenolone unlike the stroma of the LH injected ovary.

Effect of intravenous injection of LH on the hormone levels of follicles and stroma. Steroid hormones were measured in follicles and stroma at 1 and 4 h after intravenous injection of LH (10 μg). The major compound present at 1 h in follicles was 4-androstene-3,17-dione (Fig. 3). At 4 h, 4-androstene-3,17-dione, oestradiol acetate, progesterone and pregnenolone were increased. The stroma contained more of oestradiol acetate, progesterone, 4-androstene-3,17-dione and pregnenolone at 1 h compared to that at 4 h. Oestrone acetate was found to be low both in the follicles and stroma.

Steroid hormone levels in follicles, corpora lutea and stroma at different stages of ovarian function. Endogenous levels of hormones present in follicles and stroma at oestrous, 1 and 6 h after LH and in stroma and corpora lutea at 48 h after LH were compared (Fig. 4). During oestrous, the isolated follicles contained testosterone, its acetate and 4-androstene-3,17-dione. At 1 h of LH injection, no change in the pattern was observed, while at 6 h the follicles showed increased concentrations of all steroids except testosterone and its acetate. The stroma at oestrous had more of oestradiol acetate and 4-androstene-3,17-dione, compared to the other hormones. At 1 h after LH administration, the increase was mainly in the levels of progesterone and testosterone and to a lesser degree in testosterone and oestrone acetates. All the hormones dropped to low levels at 6 h except progesterone and oestrone acetate.

The corpora lutea contained more of testosterone acetate, oestradiol acetate, progesterone, dehydroepiandrosterone and pregnenolone compared to the

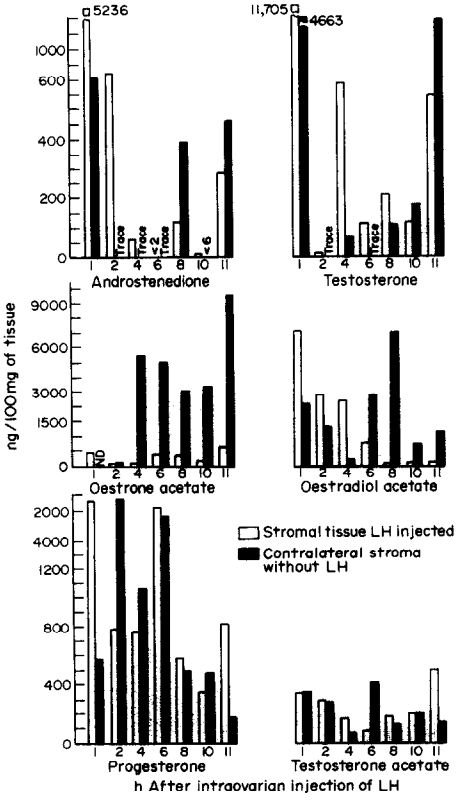

Fig. 2. Steroid hormone content of stroma at 1, 2, 4, 6, 8, 10 and 11 h after intraovarian injection of LH.

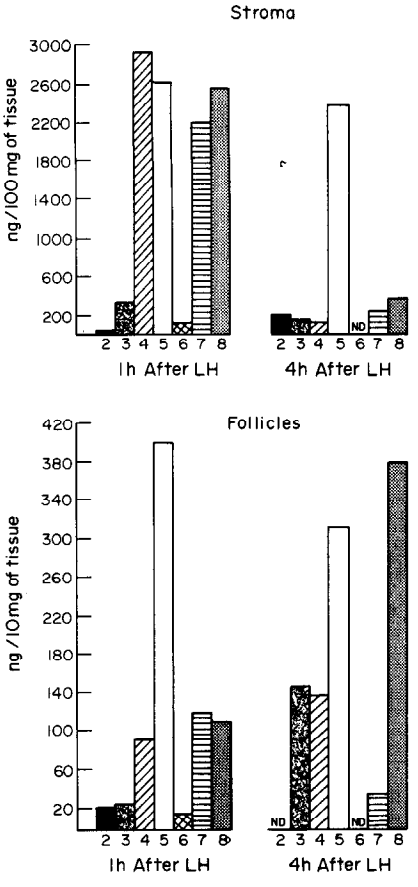

Fig. 3. Steroid hormone content of follicles and stroma at 1 and 4 h after intravenous injection of LH 2 = oestrone acetate; 3 = oestradiol acetate; 4 = progesterone; 5 = 4-androstene-3,17-dione; 6 = dehydroepiandrosterone; 7 = testosterone; 8 = pregnenolone.

hormone content of follicles. Testosterone and 4-androstene-3,17-dione were not detectable at the sensitivity of the method. The stroma of luteal phase ovary contained less of testosterone acetate and dehydroepiandrosterone than the corpora lutea. Progesterone was undetectable. Oestradiol acetate was, however, present in greater concentrations compared to other hormones in stroma.

DISCUSSION

Intraovarian injections of LH at 1 μg dose selectively induced ovulation [3] of the follicles of the injected ovary within 11 and 12 h. Analysis of hormonal content of follicles and stroma in the present study, at 1, 2, 4, 6, 8, 10 and 11 h presented differential response of these cellular compartments of the ovary to LH.

The ovarian levels of progestins and oestrogens and testosterone [4–7, 11–14] have been shown to be at peak values within 1.5 and 3.0 h of coitus or LH injection. It is not known whether these increased levels are due to the contribution by follicles or stroma or by both. Our results demonstrated that the stroma at 1 h after LH administration had higher concentrations of progesterone, testosterone, 4-androstene-3,17-dione and oestradiol acetate. At the same time interval, the follicles contained low levels of hormones. It may be argued that intraovarian injections given directly into the stromal tissue may have been responsible for the increased steroidogenesis at 1 h. Progestins were reported to be higher in stroma at 2 h after *in vivo* injection of LH [13] which further supported these findings. The levels observed at 1 and 4 h after intravenous injection of LH also indicated similar pattern in steroids of stroma as shown after intraovarian injections of LH. It thus seems probable that the ovarian effluent levels reflect changes in the stromal compartment of the ovary.

The presence of testosterone in follicles and stroma and its increased concentrations in the latter tissue in response to intraovarian injections of LH at 1 and 2 h may have physiological significance in the process of ovulation. Similar peak levels of testosterone in ovarian vein blood at 1.5 and 3.0 h post-coitus were demonstrated by Hilliard *et al.*[11] and Younglai[12].

Unlike stroma, the follicles responded to LH at 4 and 6 h with a gradual increase in the levels of progesterone, oestrone acetate and oestradiol acetate. Younglai[15] observed a similar slow response of thecal cells at 5 h post-coitus in the levels of oestrone and oestradiol-17β. A second rise in progesterone and testosterone acetate concentrations in the follicles at 11 h was never reported earlier. These levels probably reflect the enhancing luteinization, since in the present study corpus luteum also shows the presence of increased amounts of these hormones. The minimum levels of hormones observed at 8 h after LH in both follicles and stroma were in agreement with other findings [7, 11–13].

Attempts to measure oestrone and oestradiol-17β in these tissues were not successful due to the limitations of the sensitivity of the method. This, however, led to the finding of unknown compounds in phenolic steroid fractions, the peak heights of which on g.l.c. varied at different time intervals after intraovarian injection of LH in a definite pattern. The chances of esterification are less compared to saponification of the esters because of the high concentrations of sodium hydroxide used during processing of tissues. As free oestrogens were not detectable by g.l.c., methylation of phenolic steroid fraction was discontinued in later experiments.

The phenolic steroids thus isolated were identified as oestrone and oestradiol acetates from their mobilities on t.l.c. and g.l.c., enzymatic hydrolysis to free steroids and absorption maxima of oestradiol acetate in ultraviolet light. Since Forchielli *et al.*[16] and Kase *et al.*[17] reported the presence of testosterone acetate in biological tissues, no further evidence was presented. With the identification of oestrogen acetates in rabbit ovary, the presence of testosterone acetate was also considered. The presence of steroid acetates

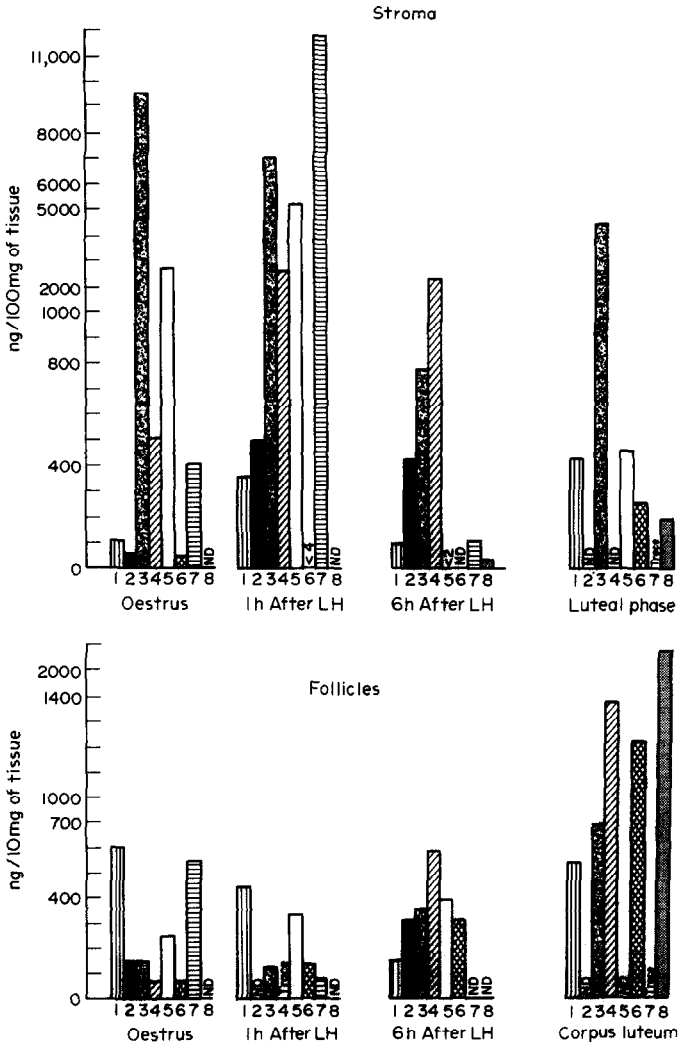

Fig. 4. Steroid hormone content of follicles and stroma at oestrus, 1 and 6 h after intraovarian injection of LH and of corpora lutea and stroma of the luteal phase. 1 = testosterone acetate; 2 = oestrone acetate; 3 = oestradiol acetate; 4 = pregesterone; 5 = 4-androstene-3, 17-dione; 6 = dehydroepiandrosterone; 7 = testosterone; 8 = pregnenolone.

was further confirmed by (i) direct identification of oestradiol acetate on t.l.c. plate after spraying with sulphuric acid, (ii) their mobilities on t.l.c. and g.l.c., (iii) enzymatic hydrolysis to corresponding free steroids and their nature on t.l.c. and g.l.c. systems, (iv) incorporation of labelled precursors into acetates and their hydrolysis free labelled steroids, (v) crystallisation to constant specific activity of labelled free and esterified compounds and (vi) infra-red spectra of testosterone and oestradiol acetates as reported earlier [8].

The changes in the pattern of oestradiol acetate in follicles and stroma in response to LH could be correlated with the reported levels of oestradiol-17β in thecal cells [15] and ovarian vein blood [4, 5]. In follicles and stroma of the contralateral ovary, oestrone acetate was, however, the major compound without demonstrating augmented changes in oestradiol acetate as in injected ovary. At the dose levels of LH injected, there was no ovulation in the contralateral ovary and the presence of oestrone acetate in large quantities may have some relation to it.

Corpora lutea contained high concentrations of testosterone acetate and oestradiol acetate, in addition to progesterone, pregnenolone and dehydroepiandrosterone. According to Telegdy and Savard [18] the rabbit corpus luteum had no capacity to synthesize oestrone and oestradiol-17β. However, these compounds were reported to be necessary for the maintenance of corpus luteum function [19, 20] and pregnancy [21]. Thus, oestradiol acetate present in the corpus luteum may be responsible for its growth and maintenance. Since the stroma of luteal phase also contained oestradiol acetate, whether the compound is a biosynthetic product of corpus luteum or contributed by the surrounding stromal tissue needs further investigation.

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