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In vitro Synthesis of Steroids by Experimentally Induced Cystic Ovaries.* (30027)

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Spontaneous ovarian cysts in women(1) and domestic animals(2,3) are associated with symptoms of endocrine dysfunction which have been attributed to abnormal ovarian and adrenal steroid secretion. *In vivo* and *in vitro* analyses of human(4) and bovine(3,5) cystic ovarian tissue suggested an increased steroidogenesis but an inability to convert C_{19} intermediates to estrogens with resultant accumulation of androgens.

Ovaries containing cystic follicles can be induced by administration of human chorionic gonadotropin to hypothyroid rats(6). Once formed, experimental ovarian cysts do not regress despite correction of the hypothyroid state, thus providing a means of studying a type of cystic ovarian disease under controlled experimental conditions. An attempt was made to analyze *in vitro* steroid production of cystic ovaries experimentally induced in rats.

Materials and methods. Female Sprague-Dawley rats weighing 60-65 g were used. Ovarian cysts were induced by adding 0.04% propylthiouracil (PTU) to the drinking water for 30 days and injecting 10 i.u. of human chorionic gonadotropin (HCG) subcutaneously daily for the last 20 days. Euthyroid rats received a similar treatment with HCG but did not develop cystic ovaries. Uninjected hypothyroid and euthyroid rats served as controls.

One ovary from each animal was incubated with ^{3}H-pregn-5-ene-3β-ol-20-one (1c/mM) while the other was incubated with ^{3}H-androst-4-ene-3,17-dione (3c/mM). To each 800 mg of sliced ovarian tissue in a 50 ml Erlenmeyer flask was added 10 ml Krebs-Hensleit buffer (pH 7.4) with 200 mg per 100 ml glucose and labelled precursor (1.11×10^6 dpm) in 0.1 ml propylene glycol. Flasks were incubated in an atmosphere of 95% oxygen and 5% carbon dioxide at 37°C with constant shaking for a total of 12 hours with a change of medium and addition of fresh precursor each 4 hours. Media from all flasks of one group were pooled, extracted and separated into nonphenolic, aqueous, and alkaline fractions(7).

Chromatography was carried out on Whatman No. 1 paper in the following systems: (A) petroleum ether 5/methanol 4:water 1; (B5) benzene 10/methanol 5:water 5(8); benzene 1:hexane 1/formamide(9); (E4) isooctane 500/t-butanol 225:methanol 225: water 50; (E2B) isooctane 500/t-butanol 250: water 450(10).

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TABLE I. *In vitro* Synthesis of Ovarian Steroids.

Treatment	Ovarian wt (mg/100 g)	Pregnenolone-H^3 precursor dpm/g ovary			Androstenedione-H^3 precursor dpm/g ovary			
		Mg tissue incubated	20αOH prog	Prog	Mg tissue incubated	Estradiol-17β	Estrone	Estriol
None	28.3 ± 1.1	533.5	136,352	50,084	545.0	—	—	—
PTU	21.7 ± 1.1	465.9	64,258	22,486	446.6	—	—	—
HCG	127.9 ± 14.7	1974.5	176,692	22,585	1993.5	60,261	33,937	23,134
PTU + HCG	329.7 ± 27.6	4274.7	332,041	22,061	5439.0	5,700	—	—

PTU = propylthiouracil. HCG = human chorionic gonadotropin (10 I.U./day).
PTU given for 30 days, CG injected last 20 days.

After location of standards the lane in which the sample was run was trimmed to a width of 2 inches and scanned for radioactivity in a 4 pi windowless gas flow counter (Nuclear Chicago model 1036) coupled to an integrating recorder.

Progesterone was identified by chromatography in systems (A) and (E4), by resistance to acetylation(11), and by reduction to a steroid having an Rf identical to 20β-hydroxypregn-4-en-3-one(12). The identity of 20α-hydroxypregn-4-en-3-one was established after chromatography in systems (A) and (E4), mobility of the acetylated product, and oxidation to a steroid having the mobility of progesterone(11). Estrogens in the alkaline fraction of the extract had the mobilities of estradiol-17β and estrone in (A), (E4), (E2B), and formamide/benzene:hexane. A steroid in the aqueous fraction of the extract had the mobility of estriol in systems (B5), (E2B) and formamide/benzene:hexane.

Radioactivity was determined quantitatively in a Packard Tricarb liquid scintillation counter. After addition of internal standard all samples were corrected for efficiency and values expressed as disintegrations per minute.

Results. When pregnenolone-H^3 was used as precursor chromatography of the nonphenolic fraction yielded 2 major peaks of radioactivity identified as progesterone and 20α-hydroxypregn-4-en-3-one. Incubates of ovaries from untreated rats yielded 20α-hydroxypregn-4-en-3-one and progesterone in a 2.7/1 ratio. Hypothyroidism depressed ovarian size ($p<.001$) and yield of each progestin to 50% oi control values but the ratio of the 20α-reduced to the oxidized form of progesterone was unaltered (2.9/1).

HCG treatment of euthyroid rats for 20 days induced formation of corpora lutea and a 4-fold increase in ovarian weight ($p<.001$). Progesterone synthesis per unit tissue was 50% of untreated control levels but 20α-hydroxypregn-4-en-3-one yield increased. The resultant ratio of 20α-hydroxypregn-4-en-3-one to progesterone was 8/1.

HCG treatment of hypothyroid rats resulted in a 15-fold increase in ovarian size which could be attributed mainly to large, fluid-filled follicles, but corpora lutea were also present. Tissue from these cystic ovaries showed a marked enhancement of 20α-reduction so that the ratio of 20α-hydroxypregn-4-en-3-one to progesterone increased to 15/1.

The radioactivity in the phenolic steroid fractions was not sufficient to permit identification of estrogens when pregnenolone-H^3 was used as precursor. When androstenedione-H^3 was used as precursor estrogens were found in incubates of ovaries from gonadotropin-injected rats but not from euthyroid or hypothyroid rats. Although less tissue was incubated from animals which had received no gonadotropin, a yield of estrogen similar to HCG-stimulated ovaries would have been within the limits of sensitivity (Table I).

Luteinized ovaries from euthyroid, HCG-injected rats produced *in vitro* estradiol-17β, estrone, and estriol. Only estradiol-17β was detected in incubates of cystic ovaries and at only 1/10 the level found in euthyroid controls.

Discussion. When rat ovarian tissue is incubated with labelled pregnenolone, major products of steroidogenesis are progesterone and 20α-hydroxypregn-4-en-3-one. Synthesis of progesterone from pregnenolone has not been previously noted in the rat but is con-

sistent with the presence of 3β-hydroxysteroid dehydrogenase in ovarian tissue in this species(13). Progesterone and the 20α-reduced derivative are also produced when rat ovarian tissue is incubated with acetate(14).

Under the conditions of these experiments androstenedione was not synthesized from pregnenolone. Others using progesterone as precursor failed to identify androstenedione when luteinized ovarian tissue from the rat was incubated(14). However, testosterone is produced in large quantities by ovarian tissue from testosterone-sterilized rats(15). Thus, the rat may be a species in which the small quantities of C-19 intermediates produced are metabolized to subsequent products so rapidly that, except under certain experimental conditions, androgens are not found in incubation media. Testosterone is metabolized to estradiol-17β, estrone, and estriol by incubates of rat ovarian tissue(16). Since androstenedione also appears to be a suitable estrogen precursor, a competent 17β-dehydrogenase enzyme must be present in ovarian tissue from this species. Thus, the pathway of ovarian steroidogenesis in rodent ovaries is similar to that in man: acetate--- pregnenolone--- progesterone (20α-hydroxypregn-4-en-3-one)--- testosterone (androstenedione)--- estrogen.

Fluid in which ovaries from untreated control rats were incubated contained 2.7 times more 20α-hydroxypregn-4-en-3-one than progesterone. This ratio correlates approximately with that observed in ovarian venous blood during the estrous cycle(17) and suggests that ovarian steroidogenesis *in vitro* may be an indication of relative quantities of steroids secreted *in vivo*. It is well known that hypothyroidism in young animals delays maturation, disrupts estrous cycles, and impairs fertility(18). Ovaries from rats treated with propylthiouracil are smaller and also produce less steroid per unit tissue although the ratio of progesterone to 20α-hydroxypregn-4-en-3-one is similar to that in euthyroid controls.

HCG stimulates the entire steroidogenic pathway in slices of human corpus luteum (19). In euthyroid rats, HCG *in vivo* induces formation of corpora lutea and, *in vitro,* tissue from these ovaries produces large quantities of estrogen and progestin; however, the 20α-reduced form of progesterone is predominant. Progesterone and 20α-hydroxypregn-4-en-3-one are interconvertible in the rat but the direction of the equilibrium may be altered by a combination of gonadotropins which induces ovulation(20).

Cystic ovarian tissue from HCG-injected hypothyroid rats produces *in vitro* even greater quantities of 20α-hydroxypregn-4-en-3-one than luteinized ovaries from euthyroid rats. Depletion of ascorbic acid(21) and increased incorporation of acetate into cholesterol(22) suggest that steroidogenesis is enhanced in cystic ovarian tissue *in vivo* also. The biological significance of 20α-hydroxypregn-4-en-3-one is not yet apparent; however, increased 20α-hydroxysteroid dehydrogenase activity is associated with the presence of large or freshly ruptured follicles (20) and may reflect a preovulatory shift in enzymes. Thus, polycystic ovaries may be arrested in a preovulatory state as regards synthesis of 20α-hydroxypregn-4-en-3-one.

Increases in ovarian 20α-hydroxypregn-4-en-3-one parallel increases in estrogen synthesis during the estrous cycle(20) and also in HCG-injected euthyroid rats; however, estrogen *in vitro* or *in vivo* does not stimulate 20α-hydroxydehydrogenase activity or concentration(20). Furthermore, an increase in 20α-hydroxypregn-4-en-3-one is not invariably accompanied by an increase in estrogen synthesis. Although cystic ovarian tissue produces large quantities of 20α-hydroxypregn-4-en-3-one, the biosynthetic pathway subsequent to C_{21} intermediate and leading to estrogen synthesis is less active than in luteinized tissue from euthyroid rats. Thus, it seems likely that 20α-hydroxylation of progesterone represents an intermediate reaction in the pathway of estrogen biosynthesis.

The success with which an anti-estrogen, ethamoxytriphetol (MER-25) prevents induction of ovarian cysts in rats(23), suggests that impaired estrogen synthesis *per se* is not a factor in cyst etiology in this species but may represent a secondary change. In fact, the impairment of estrogen synthesis and accumulation of C_{21} intermediates which occurs in definitive polycystic ovaries in the rat may not be indicative of the steroidogenic

pattern during the period of cyst development.

Summary. Ovarian cysts were experimentally induced by injecting hypothyroid rats with human chorionic gonadotropin. Cystic ovarian tissue synthesized *in vitro* a large quantity of 20α-hydroxypregn-4-en-3-one but estrogen synthesis was low.

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Purification of Ovine Luteinizing Hormone-Releasing Factor by Gel Filtration and Ion Exchange Chromatography.* (30028)

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Release of hypophyseal luteinizing hormone (LH) appears to be controlled by a hypothalamic LH-releasing factor (LH-RF). Crude or partially purified rat, beef, or sheep hypothalamic extracts prepared from the stalk-median eminence (SME) region evoke the release of LH in a variety of test animals (1-4). Since the extracts are active after destruction of the median eminence region(2), an operation which eliminates neural control of LH, it is believed that they act directly on the pituitary to release LH. This belief is strengthened by the finding that microinjection of these extracts into the adenohypophysis induces ovulation in both rats(5) and rabbits(6). More recently, it has been possible to demonstrate a decline in stored LH-releasing activity in rats at proestrus(7, 8), and to observe LH-releasing activity in plasma of hypophysectomized rats(9). These observations suggest that variations in secretion rate of the LH-RF may bring about the altered rates of LH release which occur under various physiological conditions, such as the stages of the estrous cycle and alterations in gonadal hormone titers.

We have reported the purification of bovine LH-RF on a small scale by gel filtration

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