Steroid Hormone Formation in Human Ovarian Follicles *in Vitro*

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Synopsis

Ovarian follicles of 5 to 15 mm in diameter were isolated from 45 ovaries of 34 patients in the follicular and luteal phases of the cycle. Three experiments were done. In the first, follicles were minced and incubated in Krebs-Ringer bicarbonate buffer containing 1 to 2 μ Ci of testosterone-4- 14 C in the presence or absence of 100 IU human chorionic gonadotropin (hCG). In the second, minced follicles were incubated with 100 μ Ci of sodium acetate-1- 14 C under identical conditions. In the third, ten follicles from a single patient in the late proliferative stage of endometrial dating were cut in halves and incubated with 100 μ Ci of acetate-1- 14 C under identical conditions. The minced follicle preparation was capable of aromatizing testosterone-4- 14 C into radioactive estrone and estradiol in significant amounts. Incorporation of radioactive acetate into pregnenolone, progesterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, estradiol and estrone was assessed by reverse dilution analysis with recrystallization to constant specific activity. The major radioactive products formed were androstenedione and 17-hydroxyprogesterone in the latter two experiments. Dehydroepiandrosterone was one of the major steroids in the second experiment. The minor products were testosterone, progesterone and pregnenolone. Smaller, but definite incorporations of radioactive acetate into estradiol and estrone occurred in the second experiment. On histological examination, the follicles were characterized by atretic changes. This distribution pattern of radioactive acetate among the steroids was considered to represent the steroidogenic profile of unstimulated or atretic follicles.

The specialized role of the individual anatomical compartments of the human ovary in steroid hormone formation has

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Systemic names and abbreviations;

Pregnenolone: 3 β -hydroxy-5-pregnen-20-one; progesterone: 4-pregnene-3, 20-dione; 17-hydroxypregnenolone: 3 β , 17 α -dihydroxy-5-pregnen-20-one; 17-hydroxyprogesterone: 17 α -hydroxy-4-pregnene-3, 20-dione; dehydroepiandrosterone: 3 β -hydroxy-5-androsten-17-one; androstenedione: 4-androstene-3, 17-dione; testosterone: 17 β -hydroxy-4-androsten-3-one; estradiol: 1, 3, 5(10)-estratriene-3, 17 β -diol; estrone: 3-hydroxy-1, 3, 5(10)-estratrien-17-one; FSH: follicle stimulating hormone; hCG: human chorionic gonadotropin.

been determined for the corpus luteum (Hammerstein *et al.*, 1964) and for the stroma (Rice and Savard, 1966), by assessing the radioactive steroidal products formed from acetate-1- 14 C in an *in vitro* system. One of the serial studies done by Ryan and Smith (1961) has indicated a definite role for the follicle in estrogen production. However, the follicular tissues used in their study were obtained from a patient who had received a large dose of ovine FSH prior to surgery. The preoperative treatment may have altered the physiological features of follicular activity. Therefore,

analysis of steroidogenesis in the follicles without any pretreatment with gonadotropins would provide informations for another estimation of the role of the follicular compartment in the steroidogenesis of the human ovary.

The stimulatory effect of gonadotropin *in vitro* has been established for the corpus luteum (Rice *et al.*, 1964) and for the stroma (Rice and Savard, 1966), but not as yet for the follicle in the human ovary.

The present study was undertaken for two main purposes; in the first, to assess the histophysiological implication of the follicle in the steroidogenesis of the human ovary, and secondly, to examine the effect of gonadotropin *in vitro* on the steroidogenic activity of the human follicle.

Materials and Methods

Chemicals and gonadotropins

All solvents were of reagent grade and were redistilled prior to use. Radioactive materials were purchased from the New England Nuclear (Boston, Mass., USA). Sodium acetate-1-¹⁴C (61.8 mCi/mM) was used without further purification. Testosterone-4-¹⁴C (58.8 mCi/mM) was homogeneous on paper chromatograms. Tritiated steroids of high specific activity, which were used as internal standards for recovery, were purified by thin layer chromatography within a month before use. Acetic anhydride for acetylation was distilled from fused sodium acetate. Pyridine was distilled over lumps of barium oxide. These two chemicals were stored in the dark. Human chorionic gonadotropin (APL, 20,000 IU/vial) was provided by Ayerst Laboratories, New York, N.Y., USA.

Tissue preparations

Fresh ovaries were obtained from patients with regular menstrual cycles who were having laparotomy for gynecological indications in the follicular and luteal phases of the cycle. The specimens were chilled in cold physiologic saline and transported to the laboratory. Follicles of 5 to 15 mm in diameter were dissected intact using a Binoc-Loup (3× magnification) with all surrounding tissues stripped off. A small piece of tissue specimen taken from each follicle was subjected to histological examinations. Either minced or half-cut preparation of follicle tissues were employed in the following three experiments.

Experiment I: The follicular fluid of a dissected follicle was evacuated on a filter paper by needle puncture and the remaining follicle tissues were minced by a pair of small scissors and divided into two parts. Each part was weighed. One part was used for the control incubation and the other for the incubation with an addition of hCG *in vitro*. Each incubation was performed with follicular tissues from one patient at a time. Three incubations were pooled using a total of 5.0 μ Ci of radioactive testosterone as the substrate. The amount of tissue used were 318.8 mg for the control incubation and 302.8 mg for the hCG-added incubation from 5 ovaries of 3 patients aged from 21 to 46.

Experiment II: Minced follicular tissues from one patient at a time were prepared as in the first experiment and were incubated with 100 μ Ci of acetate-1-¹⁴C as the substrate. Twenty incubations from 26 ovaries of 20 patients aged from 22 to 49 were devoted to this experiment. The weight of the tissue preparation was 2643.1 mg for the control incubations and 2735.7 mg for the hCG-added incubations. Totally 2.0 mCi of acetate-1-¹⁴C were added as the substrate.

Experiment III: The tissue preparation used in this experiment differed somewhat from the foregoing ones. The follicles, 5 to 15 mm in diameter, from both ovaries of a single patient aged 34, were dissected. The follicular fluid was squeezed out by needle puncture. The follicles were then cut in halves; one-half for the control incubation with 310.7 mg of tissue, and the other for the hCG-added incubation with 324.7 mg of tissue. One hundred μ Ci of acetate-1-¹⁴C were used as the substrate.

Incubations

Incubations were carried out as previously described (Mason *et al.*, 1962), in 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), containing an appropriate amount of ¹⁴C precursor, at 37°C for 3 hours in a Dubnoff metabolic incubator in an atmosphere of 95% oxygen and 5% carbon dioxide, with and without the addition of 100 IU hCG per vessel. The incubation was terminated by freezing quickly the contents of the vessels. Each incubation was performed with the follicular tissues of a single patient, and each incubation vessel was stored frozen until sufficient material was accumulated for analysis.

Extraction and separation of radioactive steroids

Nine radioactive steroids formed from the acetate-1-¹⁴C were analyzed by the carrier dilution technique, reported in a previous work (Hammerstein *et al.*, 1964). Both the media and tissues in each sample pool were homogenized in a glass homogenizer; 400 μ g of nonradioactive steroid as the carrier plus 0.1 μ Ci of tritium-labelled steroids as the indicator for loss correction, were added for each steroid ex-

aminated. The mixture was extracted with ether and evaporated to dryness, then the residue was partitioned between benzene and water. The benzene-soluble material was freed from nonpolar lipid components by partitioning it between ligroin (boiling range 65–100°C) and 90% aqueous methanol. The methanolic extract was subjected to a countercurrent distribution between toluene and 1 N sodium hydroxide to separate phenolic from neutral steroids (Baggett *et al.*, 1956).

The phenolic fraction was further fractionated by two chromatographic procedures; toluene/propylene glycol for 17 hours to separate estradiol followed by ligroin/propylene glycol for 72 hours to separate estrone.

The neutral fraction was chromatographed on a paper strip for 17 hours in ligroin/propylene glycol system and zones of radioactivity were located by scanning. A polar zone I (containing testosterone, 17-hydroxyprogesterone and 17-hydroxypregnenolone), a central zone II (containing dehydroepiandrosterone), a less polar zone III (containing pregnenolone and androstenedione) and the run-off (containing progesterone) were located and collected for further separation. Zone I was rechromatographed for 6 hours in toluene/propylene glycol system to separate the area containing 17-hydroxypregnenolone from the area containing testosterone and 17-hydroxyprogesterone. The former steroid was eluted and rechromatographed for 16 hours in toluene/propylene glycol system while the latter two steroids were treated for acetylation of the hydroxysteroids. Zone II and III and the run-off were separately eluted and subjected to a similar acetylation procedure. The resulting steroid acetates and free steroids from all the four zones were separated on a thin layer plate of silica gel using benzene/ethyl acetate (3:1) system followed by scanning for radioactivity.

Acetylation and saponification

For acetylation, dried samples were treated with 0.5 ml of pyridine and 0.5 ml of acetic anhydride overnight at room temperature. The reagents were evaporated in a stream of nitrogen. For saponification of testosterone acetate, dried samples were treated with 20 ml of 5% sodium hydroxide in 50% ethanol for 24 hours at room temperature in the dark. The reaction mixtures were extracted with 60 ml of ethyl acetate three times, then the combined extracts were washed with 60 ml of water until neutralized, after which they were evaporated. The residue was applied to a silica gel column for further purification. The column was developed with methylene chloride and testosterone was eluted with 20 ml of 2% ethanol in methylene chloride.

Detection and measurement of carrier steroids

Δ^4 -3-Oxosteroids were located on the chromatograms using a shortwave ultraviolet lamp and were measured spectrophotometrically in methanol by the ultraviolet absorption at 240 nm. Visualization of the steroid standards on paper strips or thin layer plates was carried out by spraying them with Turnbull blue reagent (Barton *et al.*, 1952) for estrogens, and by a spray of phosphomolybdic acid reagent (Krichensky and Kirk, 1952) for 3β -hydroxy- Δ^5 -steroids. The estrogens were measured spectrophotometrically by their absorption in methanol at 281 nm. The 3β -hydroxy- Δ^5 -steroids were determined by colorimetry using sulfuric acid-ethyl alcohol reagent (Oertel and Eik-Nes, 1959).

Scanning and measurement of radioactivity

Areas of radioactivity on the paper chromatograms were located by scanning in a Vanguard Model 800 autoscanner. Scanning of the radioactivities on the thin layer chromatograms was done with a Varian Aerograph Thin Layer Chromatography Radio Scanner, Type LB 2007 H. Radioactive zones were eluted separately and duplicate aliquots were taken for the determination of radioactivity. All radioactivity measurements were made with a Packard Tri-Carb Liquid Scintillation Counter, Model 3375, with automatic external standardization. With an efficiency of 44.5 or 48.0% for ^{14}C and 43.0 or 43.5% for ^3H , the radioactivities were recorded as disintegrations per minute (dpm).

Identification and quantitative measurement of the radioactive steroids

Identification of the radioactive steroids formed from acetate-1- ^{14}C or testosterone-4- ^{14}C was verified by comparing their radioactivities with the authentic carrier steroid in the above procedure, as well as by demonstrating the radiochemical purity of each isolated steroid. Radiochemical purity was determined by repeated crystallization to constant specific activity as shown in Table 1, 2 and 3. The criteria for establishing radiochemical homogeneity was close agreement between the specific activities of the crystals on two successive crystallizations and or close agreement of the specific activities of the crystals and the residual mother liquor of the final crystallization. Radiochemical homogeneity of the radioactive testosterone formed from acetate-1- ^{14}C was further established by demonstrating the constancy of the specific activity before and after the saponification procedure. The radioactivity associated with each steroid carrier was corrected for the recovery and was further adjusted to 1g tissue in wet weight. As recorded in Table 4, recovery rates of the individual steroids seem satisfactory except those of 17-hydroxypregnenolone, which showed below 20%, but of the similar order to allow comparisons between the control and the hCG-added incubations.

Table 1. Radiochemical purification of C₂₁ steroids formed from acetate-1-¹⁴C by human follicular tissues

Compounds	Exp. no.	Control or hCG	Total radioactivity dpm	Crystallization data specific activity (dpm/mg)			
				n-1		n	
				Crystals	Mother liquor	Crystals	Mother liquor
Pregnenolone	II	c	57,600	887	2,930	826	840
		h	62,600	1,030	1,750	1,130	1,140
	III	c	2,290	18.3	43.3	12.4	12.8
		h	10,900	690	274	709	738
Progesterone	II	c	390,000	1,430	4,320	1,450	1,430
		h	426,000	1,830	3,460	1,800	1,850
	III	c	13,200	62	212	65.1	71.8
		h	10,400	162	222	150	154
17-Hydroxypregnenolone	II	c	186,000	97.8	170	88.5	99
		h	215,000	119	199	121	128
	III	c	70,800	58.5	185	59.2	55.7
		h	134,000	227	326	221	226
17-Hydroxyprogesterone	II	c	38,700	1,790	2,100	1,710	1,790
		h	111,000	5,510	6,680	5,520	5,720
	III	c	45,700	2,280	2,720	2,330	2,350
		h	91,100	4,610	5,060	4,830	5,050

Table 2. Radiochemical purification of C₁₉ steroids formed from acetate-1-¹⁴C by human follicular tissues

Compounds	Exp. no.	Control or hCG	Total radioactivity dpm	Crystallization data specific activity (dpm/mg)			
				n-1		n	
				Crystals	Mother liquor	Crystals	Mother liquor
Dehydroepiandrosterone	II	c	88,600	2,170	3,710	2,090	2,120
		h	69,700	2,130	2,320	2,020	1,970
	III	c	10,600	90.6	335	82	81
		h	25,300	377	451	380	378
Androstenedione	II	c	499,000	25,700	27,800	24,900	26,300
		h	449,000	23,000	25,300	23,100	23,700
	III	c	82,800	3,360	2,950	3,280	3,210
		h	78,800	2,880	2,040	2,870	2,810
Testosterone	II	c	26,800	296	493	335	330
		h	20,900	262	576	264	291
	III	c	41,700	105	131	119	128
		h	49,600	107	331	123	128

Table 3. Radiochemical purification of C₁₈ steroids formed from testosterone-4-¹⁴C or acetate-1-¹⁴C by human follicular tissues

Compounds	Exp. no.	Control or hCG	Total radioactivity	Crystallization data specific activity (dpm) (dpm/mg)			
				n-1		n	
				Crystals	Mother liquor	Crystals	Mother liquor
Estradiol	I	c	10,400	788	1,160	786	820
		h	3,930	291	531	294	302
	II	c	5,830	98	155	91	98
		h	3,400	122	206	110	123
Estrone	I	c	8,100	744	1,260	750	801
		h	11,600	1,160	1,370	1,190	1,170
	II	c	3,650	84	485	85	94
		h	4,420	124	209	120	129

Table 4. Percent recovery of each steroid in the three experiments

Compounds	Exp. I		Exp. II		Exp. III	
	Control	hCG	Control	hCG	Control	hCG
Pregnenolone			32.9	34.5	54.7	57.9
Progesterone			71.5	67.4	36.9	44.3
17-Hydroxypregnenolone			15.5	14.3	15.3	18.4
17-Hydroxyprogesterone			22.4	28.8	41.0	26.3
Dehydroepiandrosterone			28.0	25.4	26.7	24.1
Androstenedione			67.9	64.4	44.4	41.6
Testosterone			31.6	27.2	29.5	22.4
Estrone	51.1	62.6	39.9	39.4	21.0	50.6
Estradiol	61.9	70.1	44.3	43.4	40.5	30.9

Results

Conversion of radioactive testosterone into estrogens

As indicated in Table 5, radioactive estrone and estradiol were isolated and identified in significant amounts in Experiment I, where radioactive testosterone was used as substrate. Conversion rates of testosterone-¹⁴C into estrone and estradiol were 1.17% for the control and 0.96% for the hCG-added incubation as dpm/g tissue. The yield of estrone and estradiol were calculated to be 1057 pmoles in the control and 865 pmoles in the hCG-added incubations per gram tissue.

Distribution of incorporated radioactive acetate among the steroids

In Experiments II and III the major

steroidal products formed from acetate-¹⁴C were androstenedione and 17-hydroxyprogesterone, as shown in Table 6. Dehydroepiandrosterone was one of the major steroids in addition to the above two steroids in Experiment II. Radioactive pregnenolone, progesterone and 17-hydroxypregnenolone were present as minor steroids. Significant amounts of radioactive testosterone were isolated and identified in the

Table 5. Conversion of testosterone-4-¹⁴C to estrogens by human follicular tissues

	Control	hCG	
	dpm	dpm	% of control
Estrone	54,900	73,000	133.0
Estradiol	61,700	22,500	36.5
Total estrogens*	116,000	95,500	81.9

* Combined incorporation of ¹⁴C radioactivity into estrone and estradiol.

Table 6. Incorporation of acetate-1-¹⁴C into steroids formed by human follicular tissue and the effect of hCG *in vitro*

Substrate ¹⁴ C incorporation into steroids (dpm/g tissue)	Exp. II			Exp. III		
	Control	hCG	% of control	Control	hCG	% of control
Pregnenolone	27,400	34,600	126.3	14,300	63,900	446.9
Progesterone	11,100	14,500	130.6	7,300	13,700	187.7
17-Hydroxypregnenolone	6,900	10,900	158.0	17,500	70,500	402.9
17-Hydroxyprogesterone	87,600	163,000	186.1	252,000	494,000	196.0
Dehydroepiandrosterone	122,000	171,000	140.2	24,400	78,300	320.9
Androstenedione	205,000	194,000	94.6	668,000	626,000	93.7
Testosterone	5,700	4,800	84.9	40,900	34,800	85.1
Estradiol	1,030	1,270	123.3	0	0	—
Estrone	1,390	1,870	134.5	0	0	—
Total incorporation	468,000	596,000	127.4	1020,000	1380,000	134.7

two experiments. Small amounts of radioactive acetate incorporated into estrone and estradiol were identified in Experiment II, where a complete profile of steroidogenesis was shown for the nine steroids. This profile is illustrated in Fig. 1.

Effect of hCG *in vitro*

The addition of hCG to the incubation vessels *in vitro* increased the overall incorporation of acetate-¹⁴C into the steroids by 27.4% in Experiment II and 34.7% in Experiment III as shown in Table 6. The calculated conversion rates of radioactive acetate into the steroids changed from 0.012% to 0.015% in Experiment II, and from 0.51% to 0.69% in Experiment III, after the addition of hCG *in vitro*.

However, the range of increase in the individual steroids was not uniform. No increase was observed in the incorporation of radioactive acetate into androstenedione and testosterone. Large amounts of radioactivity were accumulated in 17-hydroxyprogesterone and 17-hydroxypregnenolone after the addition of hCG in Experiments II and III. It was noticed that the incorporation of testosterone-¹⁴C into total estrogens decreased by 18.1% by the addition of hCG *in vitro* in Experiment I (Table 5).

ACETATE-1-¹⁴C INCORPORATION INTO STEROIDS IN HUMAN OVARIAN TISSUE
EFFECT OF hCG *IN VITRO*-ATRETIC

Fig. 1. Profile or spectrum of radioactive steroids formed after incubation of minced follicular tissues of ovaries from twenty patients with normal menstrual cycles (Exp. II). Tissues from one patient at a time were incubated for 3 hr in 2ml Krebs-Ringer bicarbonate medium containing 100 μ Ci acetate-1-¹⁴C. Incubations were repeated and the contents were pooled. Twenty incubations were collected in this experiment. Quantitative values were obtained by reverse isotope dilution analysis. Effect of hCG *in vitro* is shown by stippled bars. Abbreviations: Preg: pregnenolone, 17HO-Preg: 17-hydroxypregnenolone, Prog: progesterone, 17HO-Preg: 17-hydroxyprogesterone, DHA: dehydroepiandrosterone, And: androstenedione, Test: testosterone, E-1: estrone: E-2: estradiol.

Histological findings

The histology of the tissue specimens from dissected follicles showed more or less similar structures. The structure of the follicular walls was characterized by a majority of theca interna and externa cells together with a small amount of granulosa and stroma cells. The theca cells were hypertrophied with picnotic nuclei and vacuolization, but were not completely luteinized. Neither mitotic figures nor luteinization were seen with the granulosa cells, but picnosis and disintegration were sometimes recognized. The follicles under study were judged to be undergoing atretic change. The concomitant stroma cells looked like fibrocytes commonly seen in connective tissues. A representative photomicrograph is shown in Fig. 2. The endometrium taken from the patient, whose ovaries were used in Experiment III, was in the late proliferative stage with slight inflammatory change.

Discussion

The major steroidal products formed from radioactive acetate in an incubation experiment using a specific anatomical compartment of the human ovary may characterize what the histophysiological implications are in the steroid biosynthesis of the compartment. In Experiments II and III, the distribution of incorporated radioactive acetate among the steroids in human follicular tissues shows that the major products were androstenedione and 17-hydroxyprogesterone. In addition to these, dehydroepiandrosterone was one of the major steroids found in Experiment II. Similar distribution pattern for radioactive pregnenolone have been observed in experiments using isolated human follicles *in vitro* (Patwardhan and Lantheir, 1971).

The distribution pattern of radioactive acetate in the present study contrasts sharply

Fig. 2. Photomicrograph of dissected follicular tissue from a follicle in Experiment II. The tissue consists of the theca interna and externa cells together with a small amount of the granulosa and stroma cells. Thecal cells are hypertrophied with picnotic nuclei and vacuolization, but not completely luteinized. Neither mitotic figures nor luteinization are seen with the granulosa cells, but picnosis and disintegration are recognized. The concomitant stroma cells looked like fibrocytes commonly seen in connective tissues. Hematoxylin and eosin $\times 200$.

with that of human follicle tissues pretreated with ovine FSH *in vivo* (Ryan and Smith, 1961), where estrone and estradiol were the major products formed from radioactive acetate. The main difference between the two steroidogenic profiles seems to be quantitative differences in the aromatizing capacities of the two sets of follicular tissue. Conversion of radioactive testosterone to estrogens by 0.1–0.2% has been reported for human ovarian tissues *in vitro* in earlier works (Baggett *et al.*, 1956; Wotiz *et al.*, 1956). Preoperative injections of ovine FSH resulted in excessive increase in estrogens in the follicular fluid as well as in the urine (Smith and Ryan, 1962). Sequential administration of ovine FSH and hCG prior to surgery markedly increased the conversion of androstenedione to estrone and estradiol, up to 15%, by human follicular cyst linings (Smith and Ryan, 1961). Thus, the administration of gonadotropic hormones *in vivo* appears to enhance the aromatizing enzyme activity in the follicle.

In the present study no gonadotropin was given preoperatively and radioactive testosterone was converted to estrogens to a much lesser degree (approximately 0.1%) in Experiment I. It is understood that the distribution pattern of acetate- ^{14}C in the former experiment represents the steroidogenic profile of stimulated follicles. This was further verified by a recent experiment in which a similar steroidogenic pattern from radioactive acetate was observed with follicular tissues isolated from an ovary pretreated with human menopausal gonadotropin *in vivo* (Suzuki *et al.*, 1976). Although the individual follicles used in the present study may no longer be uniform in their functional states, it seems likely that the observed distribution pattern of acetate- ^{14}C among the steroids reflects the steroidogenic profile of the unstimulated follicles as a whole.

The profile obtained in the present study must further be considered in the light of follicular structure. Marked proliferation of the granulosa cells is apparent in the stimulated follicle (Ryan and Smith, 1965), which shows the structure of a preovulatory follicle. In contrast, the unstimulated follicles of the present study commonly showed atretic changes characterized by poor proliferation of the granulosa cells with occasional degenerative changes, although individual variations among the follicles seem to exist. It has been pointed out that joint action of the two types of follicle cells is required for estrogen production in the rat (Falck, 1959). A recombination of the separated granulosa and theca cells remarkably augmented the incorporation of radioactive acetate into estrogens (Ryan *et al.*, 1968), although each cell type is able to convert pregnenolone and progesterone to estrone or estradiol in the human (Ryan and Petro, 1966). Moreover, the aromatizing capacity is greater in equine granulosa cells than in the thecal cells (Ryan and Short, 1965). On the other hand, it has

been observed that human thecal cells secrete more Δ^4 neutral steroids than do the granulosa cells in tissue cultures (Channing, 1969). Major radioactive steroids on incubation of bovine follicles with acetate- ^{14}C are androgens, where follicles are composed of predominant thecal cells (Mori, 1975). The scantiness of the granulosa cells in the follicles in the present study may well be responsible for the much smaller incorporation of acetate- ^{14}C into estrogens. Thus, the observed profile for the incorporation of radioactive acetate would appear to represent the steroidogenic pattern of the atretic follicles.

Follicular tissue responded to an addition of hCG *in vitro* with an increase in the overall incorporation of approximately 30%. However, this difference has to be evaluated from the point of conversion rates of radioactive acetate into the steroids. Although steroidogenic activity may differ between the tissues employed in Experiment II and III, the conversion rates of radioactive acetate into the steroids were very low in both experiments, even after the addition of hCG *in vitro*. This makes proper estimation of the effect of the added hCG almost impossible. The reduction in conversion of radioactive testosterone to estrogens in Experiment I in the presence of hCG *in vitro* appears to be compatible with the results reported by Oakay and Stitch (1968), as interpreted by an increase in the size of the endogenous pool of testosterone and subsequent dilution of the radioactive testosterone added as substrate, following stimulation of the pathway leading to testosterone synthesis by the action of hCG *in vitro*. Further investigations are needed to assess the effect of hCG *in vitro* on follicular steroidogenesis.

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