NOTE

The Conversion of Pregnenolone-7 α -³H and Progesterone-4-¹⁴C to Estrogens by Corpora Lutea of Menstrual Cycles and Pregnancy

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Synopsis

The metabolism of pregnenolone-7 α -³H and progesterone-4-¹⁴C by human corpora lutea tissue of menstrual cycles and pregnancy was studied. In the incubations, equimolar mixtures of pregnenolone-7 α -³H and progesterone-4-¹⁴C were used as substrates. Three corpora lutea of cycles were used as minced tissue. From those corpora lutea progesterone, 17-hydroxyprogesterone and androstenedione were formed, although no estrogens were formed. One corpus luteum of cycle and one corpus luteum of pregnancy were used as homogenated tissue, and those formed estrone and estradiol as well as the same three Δ^4 -metabolites. The corpus luteum of cycle also formed testosterone.

All metabolites including estrogens showed the lower ³H to ¹⁴C ratio than the starting ratio. 17-hydroxypregnenolone in only one corpus luteum, and no Δ^5 -metabolites in the other four corpus luteum were identified. It is therefore proposed that the major pathway for estrogen formation in human corpus luteum is pregnenolone $\rightarrow$ progesterone $\rightarrow$ 17-hydroxyprogesterone $\rightarrow$ androstenedione (or testosterone) $\rightarrow$ estrone and estradiol.

The capacity of human corpus luteum to synthesize estrogens as well as progesterone is now widely accepted (Huang and Pearlman, 1963; Sweat *et al.*, 1960; Ryan, 1963a; Arceo and Ryan, 1967; Somma *et al.*, 1969; Savard *et al.*, 1965).

Although there is an indication at the present time that pregnenolone is converted to estrogens via Δ^4 -3-one intermediates in human corpus luteum (Ryan, 1963a), the pathway for steroid formation by human corpus luteum have not been completely defined. The present report is concerned with the pathway from pregnenolone to estrogens in human corpus luteum. By using equimolar mixture of pregnenolone-7 α -³H and progesterone-4-¹⁴C as substrates, and by isolating intermediates, an attempt was made

to distinguish between a pathway involving Δ^5 - β -ol intermediates to estrogen and one involving Δ^4 -3-one intermediates to estrogen or any possible combination.

Materials and Methods

Tissue preparation and incubation

Four corpora lutea were obtained on different occasions from four normally menstruating women at laparotomy, and one corpus luteum of pregnancy was obtained from the women hysterectomized for leiomyoma of the uterus. For details see Table 1.

Corpus luteum No. 1, 2 and 3—

Corpora lutea were dissected free of other ovarian tissue. Small portions of those tissue were dissected for histology and the rests were minced, weighted and divided into two for duplicate. The tissue were next put in 5 ml of Krebs-Ringer-phosphate buffer (pH 7.4). Incubations were carried out for 30 minutes at 37°C in air with constant agitation after 12.1 $\times$

Table 1. The clinical conditions and tissue preparations of five corpora lutea obtained

Corpus luteum	Age of patient	Day of cycle obtained	Weight of corpus luteum (grams)	Tissue preparation
No. 1	49 years	day 38*	0.36	Minced
No. 2	38 years	day 19	0.59	Minced
No. 3	38 years	day 22	1.1	Minced
No. 4	29 years	day 23	1.0	Homogenated
No. 5	45 years	16 week pregnancy	1.1	Homogenated

* The patient was operated at her follicular phase, but her ovary still had corpus luteum, so this corpus luteum seemed to be the last cycle's one.

Fig. 1. Scheme of extraction and purification

10⁶ dpm of pregnenolone-7 α -³H (5.5 μ g) and 2.6 × 10⁶ dpm of progesterone-4-¹⁴C (5.6 μ g) were added.

The radioactive substrates were obtained from New England Nuclear Corporation. Those two substrates were almost the same weight. The radioactive substrates were purified by paper chromatography prior to use. Progesterone-4-¹⁴C was used at its original specific activity and pregnenolone-7 α -³H was

used after dilution with pure authentic nonradioactive substance.

Corpus luteum No. 4 and 5—

Small portions of corpora lutea were dissected for histology. The rest tissue weighing 1.0 g and 1.1 g were put through a small tissue press to remove connective tissue. The tissue were next homogenated with 5 ml of buffer consisting of 0.25 M sucrose,

90% methanol and extracted three times with an equal volume of hexane. The 90% methanol was evaporated and the extract was subjected to a phenolic separation forming a neutral and a phenolic fraction (Cohen and Marrian, 1934; Engel *et al.*, 1950). The hexane fractions containing 3–7% of the radioactivities were not processed further. The neutral fraction with added carrier steroids was further fractionated into digitonide and non-digitonide fractions by digitonide treatment (Butt *et al.*, 1948). Further purification and separation of radioactive metabolites were accomplished by Bush paper chromatography and thin-layer chromatography. Paper chromatography was carried out using the systems ligroin/80% methanol and toluene: ligroin (1:2)/85% methanol. Thin-layer chromatography was carried out on silicagel G using the systems chloroform/methanol (99:1) (Hagerman and Spencer, 1964), and n-hexane/ethyl acetate (75:25) (Lisboa, 1963, 1964). The elutes from the paper and thin layer chromatography were identified by recrystallization to constant specific activity following addition of 15–25 mg of appropriate reference materials. The phenolic fraction was further purified by a repeated phenolic separation and subjected to Bush paper chromatography in toluene: ligroine (1:2)/85% methanol. The areas corresponding to estrone and estradiol were eluted. The estradiol iso-

Extraction
Corpus luteum No. 1, 2 and 3—(Fig. 1)

Corpus luteum No. 4 and 5—(Fig. 2)

Purification (Fig. 1 and 2)

All corpora lutea were treated with the same procedure. The chloroform residue was dissolved in

Homogenated tissue (Corpus luteum No. 4, 5)

Incubated mixture
Extracted with CHCl_3 ($3 \times 30 \text{ ml}$) and
Ethylacetate ($2 \times 30 \text{ ml}$)

lated was converted to estrone by placental 17β -ol dehydrogenase (Langer and Engel, 1958). The estrone or estradiol originally formed by incubation with the ovarian luteal tissue and the estrone, converted from estradiol by the placental dehydrogenase, were methylated and then crystallized after addition of carriers. Assay of radioactivity was performed in a Packard Tri-carb scintillation spectrometer using 2 channels to measure ^{14}C and ^3H simultaneously with efficiency for ^3H of 22.3% and for ^{14}C of 50.4%. Crystallization data were considered acceptable if agreement of the specific activities of crystals and mother liquors 5% or less for both ^3H and ^{14}C .

Results

Isolation of the metabolites from neutral non-digitonide fraction

The neutral non-digitonide fraction containing carrier progesterone, 17-hydroxypro-

gesterone and androstenedione was chromatographed on a paper chromatography (ligroine/80% methanol). The radioactive peak areas were eluted and rechromatographed in the same system. The areas corresponding to progesterone and androstenedione were eluted, acetylated to eliminate contaminating compounds and run in the ligroine/80% methanol system. After additional progesterone (25 mg) or androstenedione (20 mg) was added, crystallizations were carried out until getting constant specific activity (Table 2, 3). The area corresponding to 17-hydroxyprogesterone, which was connected with origin radioactive peak in the system (ligroin/80% methanol), was eluted and re-run in the toluene : ligroine

Table 2. Crystallization of progesterone

	Corpus luteum									
	No. 1		No. 2		No. 3		No. 4		No. 5	
Number of crystallization ¹⁾	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$
N-2	3,940	37,210	8,890	35,090	33,200	27,240	10,090	4,070	17,520	8,840
	4,280	38,760	10,490	41,620	35,710	27,610	15,430	4,620	19,180	8,260
N-1	3,350	37,650	9,020	34,510	33,360	26,550	10,450	4,210	17,220	8,620
	3,830	37,410	9,360	35,260	32,810	27,650	11,340	4,150	16,520	8,280
N	3,490	36,150	8,520	34,950	32,890	26,330	9,760	4,080	17,590	8,940
	3,520	37,510	8,820	35,270	32,480	26,220	10,240	4,000	17,490	8,960
% Radioactivity ²⁾	0.14	4.5	0.51	9.2	0.87	3.4	0.20	0.38	2.5	5.9

- 1) Upper figure denotes specific activity of the crystals, lower figure denotes specific activity of the corresponding mother liquor.
- 2) Expressed as percentage conversion of incubated substrate to progesterone or unconverted progesterone following recrystallization to constant specific activity.

Table 3. Crystallization of androstenedione

	Corpus luteum									
	No. 1		No. 2		No. 3		No. 4		No. 5	
Number of crystallization ¹⁾	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$
N-2	70	90	90	80	650	270	520	290	190	110
	180	120	100	90	700	280	570	290	200	100
N-1	70	90	100	80	650	280	520	260	200	100
	90	100	90	90	670	270	570	290	180	100
N	70	90	90	80	630	270	520	260	190	100
	70	90	90	80	650	270	530	260	190	100
% Radioactivity ²⁾	0.001	0.007	0.002	0.007	0.07	0.13	0.01	0.02	0.007	0.02

- 1) Legend as for Table 2.
- 2) Expressed as percentage conversion of incubated substrate to androstenedione following recrystallization to constant specific activity.

(1:2)/85% methanol system. The area having the same mobility to that of 17-hydroxyprogesterone was eluted, acetylated to separate testosterone or other contaminating compounds, and was chromatographed again in the same system. The most radioactivity and carrier 17-hydroxyprogesterone were unchanged and again recovered together. Adding cold 17-hydroxyprogesterone (25 mg), crystallizations were performed. The specific activities of crystals and mother liquors for both ^3H and ^{14}C agreed within 5% (Table 4). There was another small peak on the 2nd paper chromatography in the system toluene : ligroine (1 : 2)/85% methanol. This

peak was more nonpolar than 17-hydroxyprogesterone, and corresponding with testosterone acetate. This peak was eluted, added carrier testosterone acetate and chromatographed in the thin layer system n-hexane : ethyl acetate (75 : 25) (6 times development). Small peak corresponding to carrier testosterone acetate was seen in corpus luteum No. 3 and 4. Crystallizations were carried out for those peaks adding cold testosterone acetate (20 mg). Although the specific activities of crystal and mother liquor did not agree within 5% in corpus luteum No. 3, the constant specific activity was got in corpus luteum No. 4 (Table 5).

Table 4. Crystallization of 17-OH-progesterone

	Corpus luteum									
	No. 1		No. 2		No. 3		No. 4		No. 5	
Number of crystallization ¹⁾	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$
N-2	160	300	450	330	9,710	3,640	19,200	10,080	5,990	3,040
	180	340	480	340	10,110	3,820	20,600	10,660	6,670	3,480
N-1	160	300	460	320	9,450	3,600	19,520	10,440	5,820	3,010
	170	320	470	330	9,470	3,590	19,620	10,830	6,050	3,100
N	150	300	450	320	9,400	3,570	19,390	10,260	5,960	3,070
	150	310	460	320	9,620	3,620	18,850	10,170	6,000	3,160
% Radioactivity ²⁾	0.006	0.06	0.05	0.16	1.1	2.0	1.6	4.2	0.94	2.3

1) Legend as for Table 2.

2) Expressed as percentage conversion of incubated substrate to 17-OH-progesterone following recrystallization to constant specific activity.

Table 5. Crystallization of testosterone isolated from incubation of No. 3 and No. 4 corpus luteum

Corpus luteum	No. 3		No. 4	
	Testosterone acetate		Testosterone acetate	
Material crystallized	Testosterone acetate		Testosterone acetate	
Number of crystallization ¹⁾	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$	dpm $^3\text{H}/\text{mg}$	dpm $^{14}\text{C}/\text{mg}$
N-2	200	60	380	170
	580	150	990	360
N-1	170	50	270	120
	260	60	900	300
N	180	50	270	120
	170	50	280	120
% Radioactivity ²⁾			0.01	0.02

1) Legend as for Table 2.

2) Expressed as percentage conversion of incubated substrate to testosterone following recrystallization of the testosterone acetate derivative to constant specific activity.

Isolation of the metabolites from neutral digitonide fraction

The neutral digitonide fraction containing carrier pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone was subjected to paper chromatography in the system ligroine/80% methanol. The radioactive peak areas were eluted and rechromatographed in the same system. Besides the starting pregnenolone, there were two peaks. The one of them was small and coincided with the carrier dehydroepiandrosterone. The other one was rather big peak at origin, which coincided with carrier 17-hydroxypregnenolone. The eluted small peak was rerun in the same system to eliminate contaminating compounds. A few radioactivities were recovered with carrier dehydroepiandrosterone in corpus luteum No. 1. Although the crystallization was carried out for this radioactivity, the constant specific activity was not seen. No radioactivity was recovered with carrier dehydroepiandrosterone in corpus luteum No. 2, 3, 4 and 5. The big origin peak was subjected to thin layer chromatography in the system chloroform: methanol (99:1) (3 times development). Some radioactivities were recovered with carrier 17-hydroxypregnenolone in corpus luteum No. 1, 2 and 4. Additional 17-hydroxypregnenolone (15 mg) was added in each radioactivity and crystallization was carried out. Only one 17-hydroxypregnenolone was identified in corpus luteum No. 1 (Table 6).

Isolation of estrogens

The purified phenolic fraction was subjected to paper chromatography in the system toluene: ligroine (1:2)/85% methanol. In the phenolic fraction of corpus luteum No. 4 and 5 incubation, two radioactive metabolites were isolated which corresponded to standard estrone and estradiol. No radioactivity corresponding to standard estrone or estradiol was isolated in corpus luteum No. 1, 2 and 3. With additional

carrier, the metabolite corresponding to estradiol in corpus luteum No. 4 was converted to estrone by a placental estradiol 17 β -ol dehydrogenase and the converted estrone was purified in paper chromatography in the system toluene: ligroine (1:2)/85% methanol. The metabolite corresponding to estrone, estradiol and the converted estrone from estradiol were purified with methyl ether formation. The radioactivities remained with the carriers through all these steps and those radioactivities were then crystallized to constant specific activity (Table 7).

Comparison of ^3H - ^{14}C ratio

The starting ^3H - ^{14}C ratio of the two substrates, pregnenolone-7 α - ^3H and progesterone-4- ^{14}C was 4.7. The ratio was calculated for each metabolite isolated, and results are presented in Table 8. Because ratios in all metabolites were lower than starting ratio,

Table 6. Crystallization of 17-OH-pregnenolone

Corpus luteum	No. 1
Number of crystallization ¹⁾	dpm ^3H /mg
N-2	430
	460
N-1	430
	440
N	420
	430
% Radioactivity ²⁾	0.018

1) Legend as Table 2.

2) Expressed as percentage conversion of incubated substrate to 17-OH-pregnenolone following recrystallization to constant specific activity.

The following trivial names have been used.

Pregnenolone = 3 β -hydroxy- Δ^5 -pregnen-20-one.

17-OH-pregnenolone = 17 α -hydroxy- Δ^5 -pregnen-3 β -ol-20-one.

17-OH-progesterone = 17 α -hydroxy- Δ^4 -pregnene-3, 20-dione.

Dehydroepiandrosterone = 3 β -hydroxy- Δ^5 -androstene-17-one.

Androstenedione = Δ^4 -androstene-3, 17-dione.

ATP = adenosine triphosphate.

NAD = nicotinamide adenine dinucleotide.

NADP = nicotinamide adenine dinucleotide phosphate.

Table 7. Crystallization of estrone and estradiol

Corpus luteum	Estrone				Estradiol			
	No. 4		No. 5		No. 4		No. 5	
Material crystallized	Estrone-3-methyl ether		Estrone-3-methyl ether		Estrone-3-methyl ether		Estradiol-17 β -3-methyl ether	
Number of crystallization ¹⁾	dpm ³ H/mg	dpm ¹⁴ C/mg	dpm ³ H/mg	dpm ¹⁴ C/mg	dpm ³ H/mg	dpm ¹⁴ C/mg	dpm ³ H/mg	dpm ¹⁴ C/mg
N-2	1,760	780	330	110	1,090	530	320	110
	2,050	1,050	390	130	1,090	580	360	110
N-1	1,870	750	320	120	1,050	450	310	110
	1,910	780	310	120	1,090	470	310	100
N	1,810	760	320	120	1,000	440	320	100
	1,800	790	320	120	1,040	450	310	100
% Radioactivity ²⁾	0.14	0.27	0.02	0.04	0.06	0.13	0.02	0.03

1) Legend as for Table 2.

2) Expressed as percentage conversion of incubated substrate to estrone and estradiol-17 β following recrystallization of the estrogen methyl ether derivative to constant specific activity.

it is apparent that progesterone-4-¹⁴C was incorporated to all metabolites to greater extent than pregnenolone-7 α -³H under these *in vitro* condition.

Table 8. ³H-¹⁴C Ratios of steroid metabolites isolated from incubations, starting ratio is 4.7

Corpus luteum	No. 1	No. 2	No. 3	No. 4	No. 5
Metabolites					
Progesterone	0.1	0.2	1.3	2.4	2.0
17-OH-progesterone	0.5	1.4	2.6	1.9	1.9
Androstenedione	0.8	1.1	2.3	2.0	1.9
Estrone				2.4	2.7
Estradiol				2.3	3.1

Discussion

In human ovarian tissue, the possibilities of several pathway for estrogen biosynthesis have been raised (Ryan, 1963b), and it has been proposed that in human corpus luteum tissue the major pathway for estrogen formation was pregnenolone $\rightarrow$ progesterone $\rightarrow$ 17-hydroxyprogesterone $\rightarrow$ androstenedione or testosterone $\rightarrow$ estradiol (Ryan, 1963a). In Ryan's report, the same amount of radioactivity (cpm) for pregnenolone-7 α -³H and progesterone-4-¹⁴C was used. Then the weight of progesterone which was used as

substrate was about 30 times as much as that of pregnenolone, because the specific activity of pregnenolone-7 α -³H was about 30 times as high as that of progesterone-4-¹⁴C. As enzymatic reaction may greatly influenced by the substrate levels, the same weight of pregnenolone-7 α -³H and progesterone-4-¹⁴C were used simultaneously in this experiment, to test there is any difference in the major pathway for estrogen formation, compared with Ryan's report (1963a). As shown in Table 8, the ratios of ³H to ¹⁴C in the corpus luteum No. 4 and 5, which were 4.7 and 4.7 in the starting materials, were changed into 2.4 and 2.0 in the isolated progesterone, 1.9 and 1.9 in the isolated 17-hydroxyprogesterone, 2.0 and 1.9 in the isolated androstenedione, 2.4 and 2.7 in the isolated estrone and 2.3 and 3.1 in the isolated estradiol, respectively. Although the ratios in estrone and estradiol of No. 5 corpus luteum were slightly higher than other isolated metabolites, the ratios in all isolated metabolites were lower than starting ratio. In the No. 1, 2 and 3 corpus luteum, the ratios in all isolated metabolites were also lower than the starting ratio. Those lower ratios suggest that the ¹⁴C-labeled progesterone is converted to estrogens in greater extent than pregnenolone-7 α -³H.

Failure to find radioactivity in 17-hydroxypregnenolone and dehydroepiandrosterone, except No. 1 corpus luteum (Table 6), probably reflects that the conversion of pregnenolone to progesterone is the first main step of metabolism of pregnenolone in human corpus luteum tissue.

It is confirmed that in human corpus luteum, the major pathway for estrogen formation was pregnenolone → progesterone → 17-hydroxyprogesterone → androstenedione (or testosterone) → estrone or estradiol.

Ability of synthesizing metabolite showed large variations in ovary by ovary. Savard *et al.* (1965) observed that human corpora lutea showed large variations in their ability of acetate incorporation into progesterone and of their response to gonadotropin. They also observed that a human corpus luteum, obtained from a woman in the proliferative phase of cycle, where the corpus luteum has apparently persisted from previous cycle, failed to respond to LH *in vitro*, but synthesized little progesterone. I had the same opportunity to examine the previous cycle's corpus luteum (Table 1), and a very low activity has been observed in that corpus luteum obtained in proliferative phase of cycle. There are probably many factors for those variation *in vitro* corpora lutea of cycle and pregnancy. Two corpora lutea showed the ability of producing testosterone, and one testosterone was identified in corpus luteum No. 4 (Table 5). The ^3H to ^{14}C ratio of identified testosterone was calculated as from Table 5. This value was almost the same as that of androstenedione. Somma *et al.* (1969) also observed that the corpus luteum produced some testosterone as well as androstenedione *in vitro* experiment. Although it is supposed that testosterone is one of the intermediates on the way to the estrogens, more estrone and estradiol are synthesized from androstenedione than from testosterone in human corpus luteum, according to the results of Fahmy *et al.*'s experiment *in vitro* (1969).

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