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CONVERSION OF 20α-HYDROXY-4-PREGNEN-3-ONE TO PROGESTERONE BY HUMAN ENDOMETRIUM *IN VITRO*

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SUMMARY

Human endometrial tissues during the reproductive cycle were incubated *in vitro* with tritium labelled 20α-hydroxy-4-pregnen-3-one. It was shown that 20α-hydroxysteroid dehydrogenase, catalyzing the oxidative conversion of 20α-hydroxy-4-pregnen-3-one to progesterone with NAD, is mainly associated with the mitochondrial fraction of endometrial cells. Histochemical studies showed that glandular cells of the endometrium have high activity while the stromal cells have only weak activity. Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in the endometrium at different phases of the menstrual cycle indicated a progressive increase from the early proliferative phase until the late secretory phase. The conversion rate in decidua was higher in the first trimester of gestation than in the latter half. In patients with primary sterility the conversion rate was very high in the endometrium. The reason for this phenomenon is unknown.

INTRODUCTION

THERE have been several investigations on the metabolism of progesterone in the human endometrium and myometrium. 20α-Hydroxy-4-pregnen-3-one has been shown to be one of the main products of metabolism of progesterone in the endometrium and myometrium during the proliferative phase of the menstrual cycle[1, 2] and in the secretory endometrium[3]. Zander *et al.*[4] demonstrated that 20α-hydroxysteroid dehydrogenase activity is present in the myometrium and that the equilibrium favours the oxidative reaction in this tissue. The present work demonstrates the conversion of 20α-hydroxy-4-pregnen-3-one to progesterone *in vitro* in human endometrium obtained during the menstrual cycle and pregnancy.

MATERIALS AND METHODS

Chemicals. Reagent grade organic solvents were redistilled before use. [1,2-^{3}H]-20α-hydroxy-4-pregnen-3-one (33·5 Ci/mmol) was purchased from New England Nuclear Corporation, Boston, Mass. and shown by thin layer chromatography in toluene–ethyl acetate (1 : 1 v/v) to be without contamination. Nonradioac-

Abbreviations used: Chlormadinone acetate; 17α-Acetoxy-6-chloro-4,6-pregnadien-3,20-dione, Norethisterone; 4-Estren-17α-ethinyl-17β-ol-3-one, Nitro-BT; 2,2′-di-(*p*-nitrophenyl)-5,5′-diphenyl 3,3-3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride.

tive carrier steroids were purchased from Ikapharm Corporation, Israel. NAD and NADP were obtained from Sigma Chemical Co. Polyvinylpyrrolidone-K90 was purchased from Katayama Chemical Co., Japan.

Tissues. The human endometrium used in this study was obtained either by curettage or by hysterotomy preceding removal of the uterus. After removal the tissue was immediately placed in ice-cold physiological saline. The endometrium was dated with respect to the menstrual cycle by the method of Noyes *et al.*[5]. Decidual tissues were obtained on laparotomy of cases of ectopic pregnancy or caesarean section or on interruption of pregnancy for medico–social reasons.

Incubation. Tissues (0·1–0·5 g of endometrium or 0·5 g of decidua) were homogenized in 4 ml of Krebs–Ringer phosphate buffer, pH 7·4 and centrifuged at 800 *g*. The supernatant was incubated with 0·125 μCi of [1·2-^{3}H]-20α-hydroxy-4-pregen-3-one, 25 μg of authentic 20α-hydroxy-4-pregnen-3-one and 4 mg of NAD in a final volume of 5 ml with shaking for 60 min at 37°C under air. Incubations were started within 30 min after obtaining the tissue. Reactions were terminated by addition of 5 vol of acetone; then the mixtures were left overnight at 0–4°C before analysis.

Extraction of steroids. Steroids were extracted as described previously[6]. Protein was removed from the incubation mixture by filtration and the aqueous acetone mixture was evaporated to the initial volume of the aqueous phase and extracted three times with 4 vol of ether–chloroform (4 : 1 v/v). The extracts were combined and evaporated to dryness and the residue was dissolved in 30 ml of 70% methanol and stored for 18 hr at − 15°C. The preparation was then centrifuged in the cold at 3000 rev./min for 15 min, the supernatant solution was evaporated to about 9 ml and extracted three times with 2 vol of ethyl acetate. The extracts were combined and evaporated to dryness.

Column chromatography. The dried extracts were chromatographed on silica gel, eluted successively with 100 ml volumes of benzene and benzene containing 5, 20, 30 and 40% ethyl acetate.

Progesterone was eluted chiefly with benzene containing 20% ethyl acetate and partly with benzene containing 30% ethyl acetate, while 20α-hydroxy-4-pregnen-3-one was eluted partly with benzene containing 30% ethyl acetate and chiefly with benzene containing 40% ethyl acetate[7].

Thin-layer chromatography. The eluates containing progesterone and 20α-hydroxy-4-pregnen-3-one were combined, and evaporated to a small volume. They were then chromatographed on a 0·3 mm thick layer of silica gel GF (Merck) in toluene–ethyl acetate (1 : 1 v/v). Steroid products were located on the chromatogram using a thin layer chromatogram scanner with an automatic recorder and from their ultraviolet absorption employing a modified form of Toshiba scanner.

Determination of steroids. Progesterone and 20α-hydroxy-4-pregnen-3-one were determined quantitatively from their optical densities at 220, 240 and 260 nm in an ethanol solution. The value of the optical density at 240 nm was corrected using Allen's formula[8]. The radioactivity was measured with a liquid scintillation spectrometer. Radiochemical purity was measured as described previously[6]. As shown in Fig. 1, in a preliminary experiment under the incubation condition described above no radioactive metabolites of labeled 20α-hydroxy-4-pregnen-3-one other than progesterone were detected with a thin layer scannogram. Therefore, the recovery of radioactivity was calculated as follows.

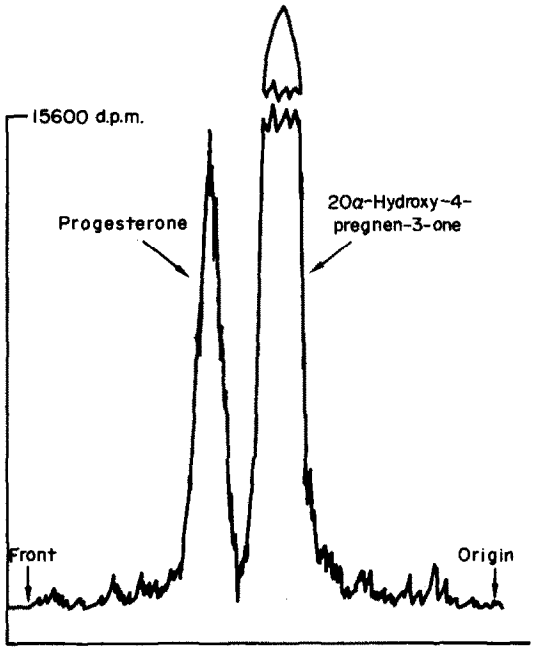

Fig. 1. Thin-layer chromatogram of products of 20α-hydroxy-4-pregennen-3-one formed in endometrial tissue (Radioscan). Solvent–toluene–ethyl acetate (1 : 1 v/v).

$$\text{Recovery } (\%) = \frac{P + S'}{S} \times 100$$

P: radioactivity of product, progesterone.
S: radioactivity of substrate, 20α-hydroxy-4-pregnen-3-one.
S′: radioactivity of unchanged substrate, unchanged 20α-hydroxy-4-pregnen-3-one.

The recovery of radioactivity in 27 samples was 80·32±3·53 (mean±S.D.).

Histochemical studies on 20α-hydroxysteroid dehydrogenase activity in endometrium. A modification of the method of Balogh [9] was used. Fresh blocks of endometrium were frozen on dry ice. Frozen sections of 8 μm thickness were cut with a rotary microtome in a cryostat (−18°C). The sections were placed on coverslips, thawed and kept at room temperature for 30 min. They were then incubated aerobically at 37°C for 60 min in medium containing 5 mg of Nitro-BT, 5 mg of NAD and 10 mg of EDTA in 2 ml of 0·05 mM phosphate buffer, pH 7·4. In order to control diffusion of the enzyme, 2 ml of a 5% polyvinylpyrrolidone solution (in 0·05 mM phosphate buffer, pH 7·4) were pipetted into the medium. Finally, 1 ml of N,N-dimethyl formamide containing 5 mg of 20α-hydroxy-4-pregnen-3-one was stirred into the incubation solution. Control incubation solution was prepared in an identical manner but excluded steroid and NAD. The incubation was terminated by fixing the sections in neutral 10% formalin at room temperature for 1 h. The sections were briefly rinsed in physiological saline solution, mounted in glycerol and examined microscopically.

RESULTS

Time course of the reaction

The time course of the enzyme reaction is shown in Fig. 2. Almost linear increase of progesterone production was observed for the first 60 min, and then

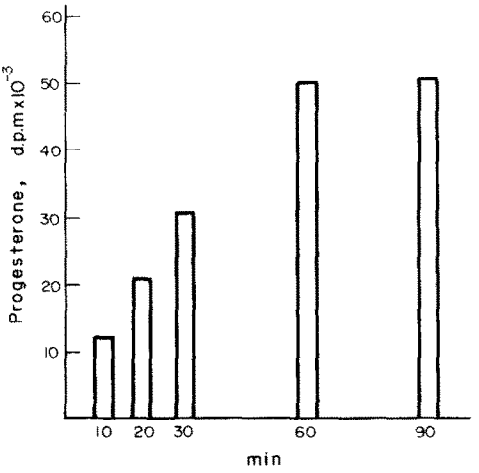

Fig. 2. Time course of progesterone production. Each flask contained 25 μg of 20α-hydroxy-4-pregnen-3-one (244000 d.p.m.), the supernatant fraction (800 g of 0·2 g of endometrium and 4 mg of NAD in 5 ml of Krebs–Ringer phosphate buffer, pH 7·4, and was incubated at 37°C. The abscissa shows radioactivity of progesterone produced per incubation mixture.

the rate of production decreased. Accordingly, 1 h was chosen as the standard time of incubation.

Effect of enzyme concentration on activity. Figure 3 shows the effect of enzyme concentration (wet wt of tissue) on the reaction. In the presence of excess substrate the reaction was proportional to the weight of tissue (from 50 to 500 mg) in the reaction mixture.

Cofactor requirements for enzyme activity. The results are shown in Table 1. Optimum activity was obtained in the presence of NAD as cofactor. The quantity of progesterone produced reached a plateau when 2–8 mg of NAD were added. Accordingly, 0·1–0·5 g of wet wt equivalents of the tissue homogenate were incubated with 4 mg of NAD.

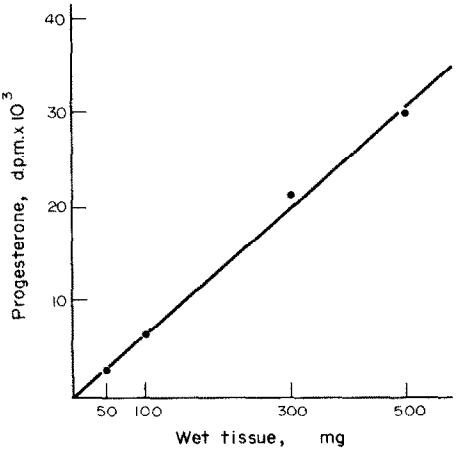

Fig. 3. Effect of enzyme concentration on progesterone production. Conditions were as for Fig. 2, except that the concentration of endometrial tissue was varied and mixtures were incubated for 1 h. The abscissa shows radioactivity of progesterone produced per incubation mixture.

Table 1. Cofactor requirements for enzyme activity

Cofactor added		Progesterone formed		
		μg	d.p.m.	d.p.m./μg
None		1·7	17300	10200
NAD,	2 mg	5·9	60200	10200
NAD,	4 mg	5·6	60400	10800
NAD,	8 mg	5·8	58900	10200
NADP,	4 mg	2·6	24900	9580

Flask content: 25 μg of 20α-hydroxy-4-pregnen-3-one (244000 d.p.m.), the supernatant fraction (800 g) of 0·5 g of endometrium, cofactor as indicated in 5 ml of Krebs–Ringer phosphate buffer, pH 7·4. Incubation was carried out for 1 h at 37°C.

Localization of enzyme activity. (*a*) *Differential centrifugation.* Endometrial tissue was fractionated by differential centrifugation (Fig. 4). The activity of the enzyme capable of oxidizing the 20α-hydroxy group of 20α-hydroxy-4-pregnen-3-one (20α-hydroxysteroid dehydrogenase) in these fractions are shown in Fig. 5. The activity was mainly associated with the mitochondrial fraction of the endometrial cells. (*b*) *Histochemical studies.* Intense 20α-hydroxysteroid dehydrogenase activity was demonstrated in the glandular cells of the endometrium while the stromal cells showed only weak activity (Fig. 6).

Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in the endometrium during the menstrual cycle. Results are shown in Table 2. The rate of conversion of 20α-hydroxy-4-pregnen-3-one to progesterone was low in the endometrium in the proliferative phase, but then gradually increased. It was noted in an endometrium with double corpora lutea in the early secretory phase the rate of conversion was very high (28·5%).

Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in decidua. In this study, 0·5 g samples of decidual tissues were used for incubation. The results are seen in Table 3. The rates of conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in specimens of decidua in early pregnancy, including ectopic pregnancy, were within the range corresponding to the lower levels observed in the endometrium in the secretory phase. The rate of conversion in the latter half of pregnancy decreased to the level of that of endometrium in the proliferative phase.

Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in secretory endometrium in cases of sterility and repeated abortion. Results are seen in Table 4. The rate of conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in the secretory endometrium in cases of sterility was about 2–3 times that of nonsterile patients, while that of patients suffering from repeated abortion was not significantly different from the latter.

Effects of gestagens and/or estrogens on 20α-hydroxysteroid dehydrogenase in secretory endometrium in vitro. To examine the direct action of steroids on the enzyme activity, 200 μg of the steroids were added to the standard reaction mixture. As shown in Table 5, the activity of 20α-hydroxysteroid dehydrogenase was not affected by chlormadinone acetate (C_{21} gestagen) but was inhibited considerably by norethisterone (19-norgestagen).

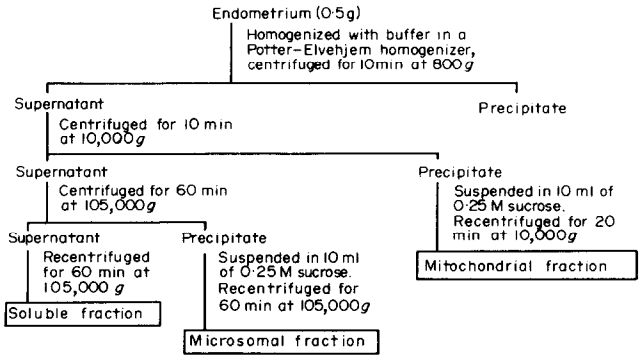

Fig. 4. Subcellular fractionation of endometrial tissue.

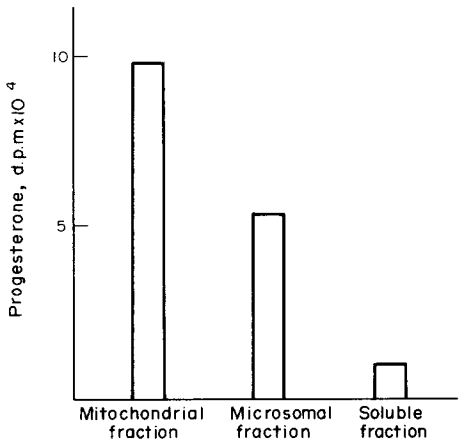

Fig. 5. Distribution of enzyme activity in subcellular fractions. Each flask contained 25 μg of 20α-hydroxy-4-pregnen-3-one (978000 d.p.m.), 4 mg of NAD and the above fractions of 0·5 g of endometrium in 5 ml of Krebs–Ringer phosphate buffer, pH 7·4, and was incubated for 1 h at 37°C. The abscissa shows radioactivity of progesterone produced per incubation mixture.

The progesterone isolated after each incubation was recrystallized repeatedly with authentic progesterone until it showed a constant specific activity (Table 6).

DISCUSSION

20α-Hydroxysteroid dehydrogenase, catalyzing the interconversion of progesterone and 20α-hydroxy-4-pregnen-3-one, has been found in different tissues, including not only the ovary and placenta, the sites of biosynthesis of these hormones, but also the liver, the site of their metabolism, and the endometrium, their target site. Both *in vivo* and *in vitro* studies showed that the interconversion of progesterone and 20α-hydroxy-4-pregnen-3-one occurs in the human feto-placental unit[10–12]. Little *et al.*[13] demonstrated the conversion of progesterone to 20α-hydroxy-4-pregnen-3-one in the soluble protein fraction of human placenta obtained by high speed centrifugation (105,000 g). Zander[14] reported that the equilibrium of the 20α-hydroxysteroid dehydrogenase reaction in the placenta favours progesterone formation, and subsequently Sieber[15] reported that the reaction in placental tissue was stimulated by NAD and that the pH

Table 2. Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in the endometrium during the menstrual cycle

Phase of cycle (Day of cycle)	Rate of conversion (%)	Clinical condition
Proliferative		
early (5)	2·5	uterine myoma (40)*
(6)	2·9	uterine myoma (44)*
mid (9)	4·8	uterine myoma (32)*
late (13)	6·9	uterine myoma (35)*
(15)	6·1	uterine myoma (43)*
Secretory		
(18)	11·6	uterine myoma (40)*, corpus luteum (1·0 g)
(19)	28·5	cervical dysplasia (45)*, double corpora lutea (2·7 g)
(22)	18·0	myoma uteri (43)*, corpus luteum (1·7 g)
(22)	23·7	normal (27)*
(27)	26·2	myoma uteri (43)*, corpus luteum (1·7 g)

*Age of patient.

Incubation conditions: Each flask contained 25 μg of 20α-hydroxy-4-pregnen-3-one (244000 d.p.m.), the supernatant fraction (800 g) of 0·4–0·5 g of endometrium and 4 mg of NAD in 5 ml of Krebs–Ringer phosphate buffer, pH 7·4, and was incubated at 37°C for 1 h in air. Rate of conversion is expressed as % conversion of substrate per 0·5 g wet wt of endometrial tissue.

Table 3. Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in decidua

Month of pregnancy	Rate of conversion (%)
II	15·9
II (ectopic)	17·2
II (ectopic)	12·3
III	12·5
III–IV	11·6
V	3·2
VI	4·9
X	5·9
X	4·1
X	2·1

Incubation conditions were as for Table 1 using the supernatant fraction (800 g) of 0·5 g of decidua.

optimum was 7·4. Recently, Zander *et al.*[15] demonstrated that the above-mentioned activity, analogous to placental 20α-hydroxysteroid dehydrogenase activity, is also present in the myometrium in pregnancy and that the equilibrium in this tissue favours the oxidative reaction. The present investigation shows that 20α-hydroxysteroid dehydrogenase activity, measured by incubation with NAD at pH 7·4, is also present in the endometrium during the menstrual cycle.

Table 4. Conversion of 20α-hydroxy-4-pregnen-3-one to progesterone in secretory endometrium of sterile patients* and those showing repeated abortion

Day of cycle	Rate of conversion (%)	Clinical condition
19	83·3	primary sterility (30)**
20	56·4	primary sterility (30)**
21	56·5	primary sterility (34)**
22	74·2	primary sterility (43)**
24	26·5	repeated abortion (27)**
26	34·5	repeated abortion (32)**

*The term "primary sterility" used in our paper is applied to the patients who have normal values of all the necessary procedures (BBT, tubal test, endometrium, semen, post-coital test).

**Age of patient.

Incubation conditions were as for Table 1 using the supernatant fraction (800 g) of 0·1–0·3 g of endometrium.

Table 5. *In vitro* effects of gestagens and, or estrogen on 20α-hydroxysteroid dehydrogenase in the secretory endometrium

Experiment (Day of cycle)	Hormone added (μg)	Rate of conversion (%)
1. (25)	control	30·3
	chlormadinone acetate (200)	31·7
	norethisterone (200)	19·7
	norethisterone with 5% mestranol (200)	18·3
2. (24)	control	32·5
	chlormadinone acetate (200)	26·7
	norethisterone (200)	9·4
	norethisterone with 5% mestranol (200)	9·8

Incubation conditions were as for Table 1 using the supernatant fraction (800 g) of 0·25 g of endometrium.

Endometrial tissue was fractionated by differential centrifugation (Fig. 5) and the activity was found to be associated with the mitochondrial fraction, as reported by Zander. There are no previous reports of histochemical studies on 20α-hydroxysteroid dehydrogenase activity in the human endometrium. In this work histochemical examination, using Nitro-BT as an indicator showed strong 20α-hydroxysteroid dehydrogenase activity in the secretory endometrium. Most activity was seen in the glandular epithelial cells of the endometrium with weak activity in the stromal cells (Fig. 6). Thus in the present work, both subcellular fractionation and histochemical studies suggest that the 20α-hydroxysteroid dehydrogenase is mainly localized in mitochondria in glandular epithelial cells of endometrium.

As shown in Table 2, 20α-hydroxysteroid dehydrogenase activity was found in endometrial tissue during almost the entire menstrual cycle. Biochemical assay in-

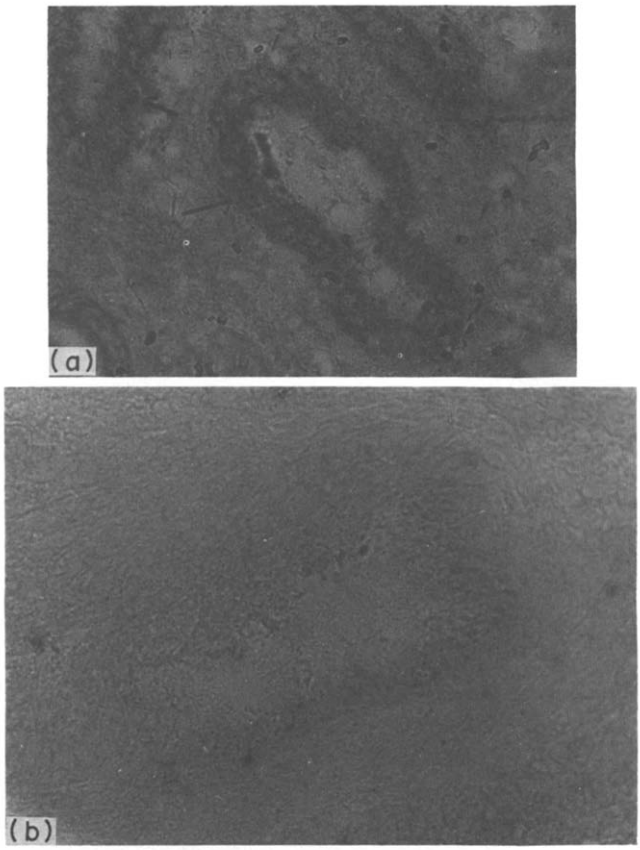

Fig. 6. Histochemical distribution of 20α-hydroxysteroid dehydrogenase in human endometrium in the secretory phase. (a) Presence of substrate and NAD. The enzyme activity is indicated by dark deposits of diformazan in glandular epithelium (arrows). Stromal cells showed only a weak reaction. (× 160). (b) Absence of substrate and NAD. No deposits of diformazan are seen in either endometrial glands or stromal cells. (× 160).

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Table 6. Recrystallization of [^{3}H]-progesterone* isolated after incubation of human endometrium with [1,2-^{3}H]-20α-hydroxy-4-pregnen-3-one

Recrystallization	S.A. (d.p.m./mg)
Initial**	12200
First: crystals	11900
mother liquor	12000
Second: crystals	10700
mother liquor	12700
Third: crystals	10700
mother liquor	11600
Fourth: crystals	10200
mother liquor	10700

*Pooled samples from the experiments in Table 1.

**Initial represents pooled eluates from thin layer chromatograms. To the pooled eluates 5 mg of carrier progesterone were added and crystals were allowed to form from the solvents shown.

The solvents used for recrystallization were (1) n-Hexane, (2) heptane–benzene, (3) acetone–pentane and (4) petroleum ether–benzene.

dicated that the activity increases during the cycle to a peak during the latter half of the secretory phase. These findings may be related to the function of the corpus luteum, which produces and secretes progestins. 20α-Hydroxysteroid dehydrogenase activity in decidua from cases of early pregnancy, including ectopic pregnancy, was similar to that of the endometrium in the secretory phase of the menstrual cycle, and a marked decrease in activity was noted from the second trimester of pregnancy until term (Table 3).

A very interesting finding in the present study was that 20α-hydroxysteroid dehydrogenase activity in the endometrium of cases with primary sterility was about 2–3 times that in normal patients, but its physiological significance is unknown. However, Hughes[16] reported that in the patients examined, the nutritional physiology of the endometrium could be classified as abnormal in approximately 80 % of the sterile patients and those showing repeated abortion. Therefore, the abnormal increase in 20α-hydroxysteroid dehydrogenase activity may be somehow related with sterility. This problem requires further investigation.

Finally, in view of the present finding that there was a remarkable difference among the conversion rates in the specimens of various conditions, it should be considered that the time period during which the rate of conversion is constant may vary from specimen to specimen. Therefore, the definite conclusion of 20α-hydroxysteroid dehydrogenase activity in the human endometrium must wait for further study.

ACKNOWLEDGEMENTS

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