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The Roles of Pregn-5-ene-3β,20α-diol and 20α-Hydroxy Steroid Dehydrogenase in the Control of Progesterone Synthesis preceding Parturition and Lactogenesis in the Rat

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1. The activity of 20α-hydroxy steroid dehydrogenase in rat ovarian corpora lutea increased at least 50-fold between 2 days before and 2 days after parturition, and then fell gradually during lactation. The activity of 3β-hydroxy Δ^5-steroid dehydrogenase decreased by 50% at parturition but remained constant at other times. 2. The 20α-hydroxypregn-4-en-3-one/progesterone concentration ratio in the ovary fell tenfold between 1 day before and 1 day after parturition, in contrast with the increase of the ratio for these steroids in plasma. 3. Pregnenolone was metabolized in intact cells or cell-free systems either to pregn-5-ene-3β,20α-diol and then to 20α-hydroxypregn-4-en-3-one by 20α-hydroxy steroid dehydrogenase and 3β-hydroxy Δ^5-steroid dehydrogenase respectively, or directly to progesterone by the latter enzyme. The relative activities of these pathways appeared to reflect the relative amounts of the two enzymes and the concentrations of their respective coenzymes NADPH and NAD^+. 4. From these and other observations it was concluded that the cessation of progesterone secretion, which precedes parturition and lactogenesis at the end of pregnancy, is partly due to the redirected metabolism of pregnenolone away from progesterone and towards 20α-hydroxypregn-4-en-3-one as the secreted end product. This is primarily the consequence of the sharp increase in the activity of 20α-hydroxy steroid dehydrogenase. This mechanism is superimposed on the already declining rate of net Δ^4-steroid release by the ovary. 5. A relationship of these pathways to subcellular compartments of luteal cells is proposed.

The withdrawal of progesterone at the end of pregnancy in the rat, which is necessary for parturition and the onset of lactation, results from two events. An abrupt switch from progesterone to 20α-HP,‡ as the major Δ^4-steroid secreted by the corpora lutea, is superimposed on the already declining rate of net Δ^4-steroid secretion from this source. This results in the disappearance of progesterone from the peripheral plasma, slowly from about 5 days before, and rapidly at about 30h before, parturition (Fajer & Barraclough, 1967; Hashimoto, Henricks, Anderson & Melampy, 1968; Wiest, Kidwell & Balogh, 1968; Kuhn, 1969*a*). During subsequent lactation progesterone once more becomes the main secreted Δ^4-steroid (Tomogane, Ôta & Yokoyama, 1969). Since the concentration of progesterone in the peripheral plasma reflects the pattern of its secretion by the ovary, which itself contains only a few minutes' supply, factors that regulate its synthesis in ovarian corpora lutea may initiate or presage the onset of parturition and lactogenesis.

The presence in ovarian homogenates of 20α-hydroxy steroid dehydrogenase capable of catalysing the reaction:

$$\text{Progesterone} + \text{NADPH} + H^+ \rightleftharpoons \text{20}\alpha\text{-HP} + NADP^+ \quad (1)$$

(Wiest, 1959) suggests that this enzyme causes the decreased rate of secretion of progesterone at parturition by converting it into 20α-HP before release into the blood (Wiest *et al.* 1968; Kuhn, 1969*a*). 20α-HP is inactive both in the maintenance of gestation (Wiest, 1968) and in the inhibition of lactogenesis in the rat (Kuhn, 1969*b*). Consistent

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‡ Abbreviations: 20α-HP, 20α-hydroxypregn-4-en-3-one; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid.

with this role, the activity of ovarian 20α-hydroxy steroid dehydrogenase increases greatly during the last 2 days of pregnancy (Fig. 1) (Wiest *et al.* 1968).

Progesterone is formed from pregnenolone by the enzyme 3β-hydroxy Δ^5-steroid dehydrogenase, which catalyses the NAD^+-linked oxidation of the 3β-hydroxyl group and a Δ^5–Δ^4 double-bond shift (Scheme 1). The enzyme, which may comprise two, as yet unresolved, catalytic proteins, resembles the corresponding enzyme of the adrenal cortex (Beyer & Samuels, 1956; Neville, Orr & Engel, 1968).

We present results on changes in activity of ovarian 20α-hydroxy steroid dehydrogenase and 3β-hydroxy Δ^5-steroid dehydrogenase at the end of pregnancy and during lactation. Evidence is given for an alternative biosynthetic route from pregnenolone to 20α-HP via pregn-5-ene-3β,20α-diol (pregnenediol) as an intermediate. It is proposed that this is, in fact, the pathway by which 20α-HP is formed in the ovary. A scheme is presented for the separate pathways of progesterone and 20α-HP synthesis involving vectorial reactions on the smooth membrane of the endoplasmic reticulum. Factors that determine the metabolic fate of pregnenolone are discussed.

METHODS

Wistar rats were used for all experiments except in the determination of ovarian progesterone and 20α-HP, where Sprague–Dawley rats were used. Litter sizes averaged about 12.

Enzyme assays. Two ovaries were homogenized in 2.5ml of 0.25M-sucrose at 0°C in a glass homogenizer fitted with a Teflon–glass pestle of clearance 0.15mm (Tri-R Instruments, Jamaica, N.Y., U.S.A.). The homogenate was centrifuged for 30min at 85000*g* and the clear supernatant was used for the assay of 20α-hydroxy steroid dehydrogenase. The sediment was rehomogenized in 2.5ml of 0.25M-sucrose and centrifuged for 5min at 150*g*, the supernatant being taken for assay of 3β-hydroxy Δ^5-steroid dehydrogenase and the inactive residue being discarded.

20α-Hydroxy steroid dehydrogenase was assayed essentially by the method of Wilcox & Wiest (1966) but with the omission of cysteine and inclusion of serum albumin. The standard reaction mixture contained glycylglycine–KOH or TES–KOH buffer, pH7.4 (40μmol), serum albumin (1mg), $NADP^+$ (0.3μmol) and enzyme (0.025–0.05ml) in a final volume of 0.70ml. This mixture was warmed to 37°C and the extinction measured at 340nm in a microcuvette of 1cm light-path for several minutes before the reaction was initiated with 0.025ml of ethanolic 20α-HP (0.5mg/ml). The extinction was measured over 2min, during which it increased linearly with time and with the amount of enzyme. Rates were completely dependent on added enzyme and $NADP^+$. No reaction was given by ethanol alone. Occasional preparations showed a significant rate before the substrate was added, and this was subtracted from the final rate. Rates were two- to three-fold higher at pH9.5–10. At pH7.4, but not at pH10, they were stimulated 50% by prior incubation of the enzyme at 0°C for 30min with 10mM-cysteine. Not understanding the significance of this effect, we decided to omit cysteine from the standard assay.

Standard 3β-hydroxy Δ^5-steroid dehydrogenase assay mixtures contained glycine–NaOH buffer, pH9.4 (40μmol), serum albumin (1mg), NAD^+ (0.3μmol), nicotinamide (10μmol) and enzyme (0.025–0.05ml) in a final volume of 0.8ml. After the reaction mixture had been warmed to 37°C the extinction change was measured at 340nm before and after the addition of 0.05ml of ethanolic pregnenolone (0.1mg/ml). Rates were proportional to time and amount of enzyme, and were completely dependent on added NAD^+. The experimentally determined coefficient 0.61 was used to correct rates at pH9.4 to those at pH7.4. No reaction was given by ethanol alone.

Steroid determinations. Progesterone and 20α-HP in rat ovaries were determined as described by Kuhn (1969*a*). Authentic carrier pregnenediol was determined fluorimetrically for measurements of specific radioactivity. The compound (0–2.5μg) was mixed with 3.5ml of concn. H_2SO_4 (Micro Analytical Reagent)–aq. 80% (v/v) ethanol (1:1, v/v) and kept for 1.25h at room temperature. The fluorescence at 500nm was then determined, with the incident light set at 436nm. Fluorescence was linearly related to the amount of steroid over this range, and the blank fluorescence was equivalent to 0.27μg. Authentic carrier 20α-HP was determined for purposes of specific-radioactivity measurement, by the extinction of its ethanolic solution at 240nm.

Steroid conversions in vitro. Radioactive steroids formed during the incubation of whole corpora lutea or of homogenates with labelled precursor were determined after their purification by chromatography. Steroids were quantitatively extracted by shaking reaction mixtures, or by homogenizing corpora lutea in incubation medium, with 2×3ml and then with 3×2ml of ethyl acetate. The appropriate ^3H- or ^{14}C-labelled steroid of known radioactivity was added to the combined extracts to correct for losses during purification. After chromatography of the extracts on columns of Florisil (Kuhn, 1969*a*) carrier steroids were added (10μg each of pregnenolone, progesterone, pregnenediol and 20α-HP) and the mixture was chromatographed by t.l.c. in solvent 1 (see below). Progesterone and pregnenediol were separately eluted with 10ml of ethyl acetate, each being mixed with further carrier pregnenolone and 20α-HP and re-run in the same solvent. Pregnenolone and 20α-HP were eluted together, mixed with further carrier progesterone and pregnenediol, and separated by t.l.c. in solvent 2. ^3H- and ^{14}C-labelled steroids were finally determined by dual-channel counting in toluene scintillator [4g of 2,5-diphenyloxazole+50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l]. There was no quenching by the added steroids.

Chromatography. Thin-layer Chromatoplates carrying 300μm layers of silica gel G were developed for 15.4cm in closed tanks lined with solvent-soaked filter paper, with the following solvents: 1, chloroform–acetone (3:1, v/v); 2, diethyl ether–dimethylformamide (99:1, v/v); 3, benzene–acetone (1:1, v/v). Reference steroids (25μg each) were run alongside unknowns and selectively sprayed with methanolic 0.05% morin (2′,3,4′,5,7-pentahydroxyflavone). Typical R_F values in solvent

1 were: pregnenediol, 0.48; 20α-HP, 0.60; pregnenolone, 0.60; progesterone, 0.72; pregnenediol diacetate, 0.77. In solvent 2, 20α-HP and pregnenolone had typical R_F values 0.37 and 0.47 respectively. For descending paper chromatography, system 1 used Whatman no. 20 paper equilibrated for 9h with methanol–water–iso-octane (9:1:10, by vol.) and developed for 24h at 28°C with the upper phase of this mixture (Eberlein & Bongiovanni, 1955). Spots were located by spraying with ethanolic 5% (w/v) phosphomolybdic acid and heating for a few minutes at 80°C. In system 2 Whatman no. 1 paper impregnated with formamide was developed at room temperature with formamide-saturated *n*-hexane (Burton, Zaffaroni & Keutmann, 1951).

Fractional recrystallization of labelled steroids was performed in the presence of 3–5mg of carrier from aq. 40% (v/v) ethanol.

Labelled 20α-HP was prepared enzymically from labelled progesterone (Kuhn, 1969*a*), and labelled pregnenediol was made from pregnenolone in the same manner. [^{14}C]Progesterone was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [^{3}H]Progesterone and [^{3}H]- and [^{14}C]-pregnenolone were from The Radiochemical Centre, Amersham, Bucks., U.K. Progesterone, pregnenolone, pregn-5-ene-3β,20α-diol and pregn-5-ene-3β,20β-diol were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Pregn-5-ene-3β,20α-diol, pregn-5-ene-3β,20β-diol and pregn-5-ene-3β,20α-diol diacetate were from the Medical Research Council Steroid Reference Collection. 20α-HP and morin were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Nicotinamide nucleotides and 20β-hydroxy steroid dehydrogenase were from Boehringer Corp. (London) Ltd., London W.13, U.K. Bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.

Thin-layer chromatograms were prepared with silica gel G (E. Merck A.-G., Darmstadt, Germany) and activated for 20min at 110°C before use. Florisil (60–100 mesh) was obtained from the Fisher Scientific Co., Fair Lawn, N.J., U.S.A. and was activated for 24h at 190°C before use.

Enzymes were assayed with a Hilger–Gilford recording spectrophotometer. Fluorescence was measured with a Zeiss spectrophotometer (PMQII) with a single monochromator (MM12) and fluorescence attachment (ZFM4). Scintillation counting was performed on a Packard Tri-Carb liquid-scintillation counter (model 314EX).

RESULTS

Changes in activities of ovarian enzymes (Fig. 1)

The two hydroxy steroid dehydrogenases were assayed in cell-free preparations of rat ovaries removed at different stages of pregnancy and lactation. Up to 2 days before parturition no 20α-hydroxy steroid dehydrogenase activity was detected, even when assayed at pH9.5, a value at which this enzyme is two to three times as active as at pH7.4. Experiments with mixed active and inactive preparations showed that no inhibitor was masking the activity. Wiest *et al.* (1968) found an activity of about 5m-units/ovary at this time in

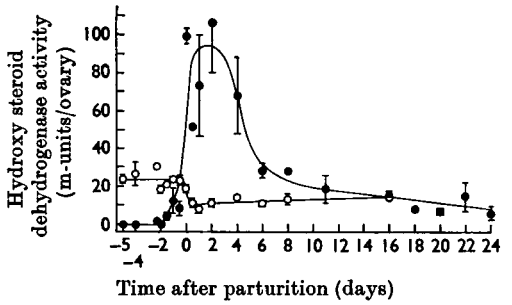


Fig. 1. Changes in activity of ovarian 20α-hydroxy steroid dehydrogenase (●) and 3β-hydroxy $^{5}\Delta$-steroid dehydrogenase (○) during pregnancy and lactation. Each point is a mean value from two to six rats (mean four). Bars indicate S.E.M. when this exceeds the diameter of the point.

Sprague–Dawley rats. The activity then rose at least 50-fold to a peak 1–2 days after parturition, and decreased gradually during lactation. The wide scatter of values just before parturition reflects the rapidly changing enzyme activity and the difficulty in relating accurately the time to parturition. Within experimental error the onset of increased activity coincided with the previously demonstrated increased 20α-HP/progesterone concentration ratio in peripheral plasma (Wiest *et al.* 1968; Kuhn, 1969*a*). A similar pattern of change was observed when the enzyme was assayed at pH9.5 (results not shown). Although Tomogane *et al.* (1969) found an increased secretion of 20α-HP near day 21 of lactation we observed no increase in 20α-hydroxy steroid dehydrogenase activity at this time. Our rats had larger litters, and the prolonged suckling may have influenced the time of this event.

3β-Hydroxy Δ^{5}-steroid dehydrogenase showed constant activity during the end of pregnancy but fell 50% at parturition to a new, constant, value. The lines of the graphs of these two enzyme activities crossed over sharply at parturition.

Changes in ovarian progesterone and 20α-HP contents (Fig. 2)

The progesterone content of the ovary fell gradually from 5 days to 1 day before parturition and then rose more than sixfold in the next 2 days. The 20α-HP content showed irregular changes. In a separate experiment with the pooled ovaries of five parturient rats, from which the corpora lutea of pregnancy were dissected, the luteal tissue accounted for 80% and 93% of the total progesterone and 20α-HP respectively.

The 20α-HP/progesterone concentration ratio (Fig. 2*b*) rose to a peak 1 day before parturition and then fell tenfold during the next 2 days. This fall

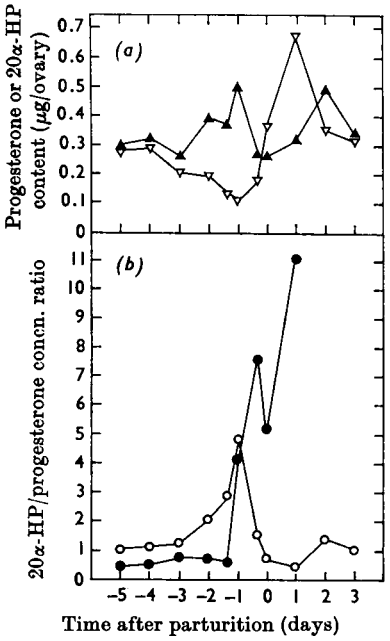


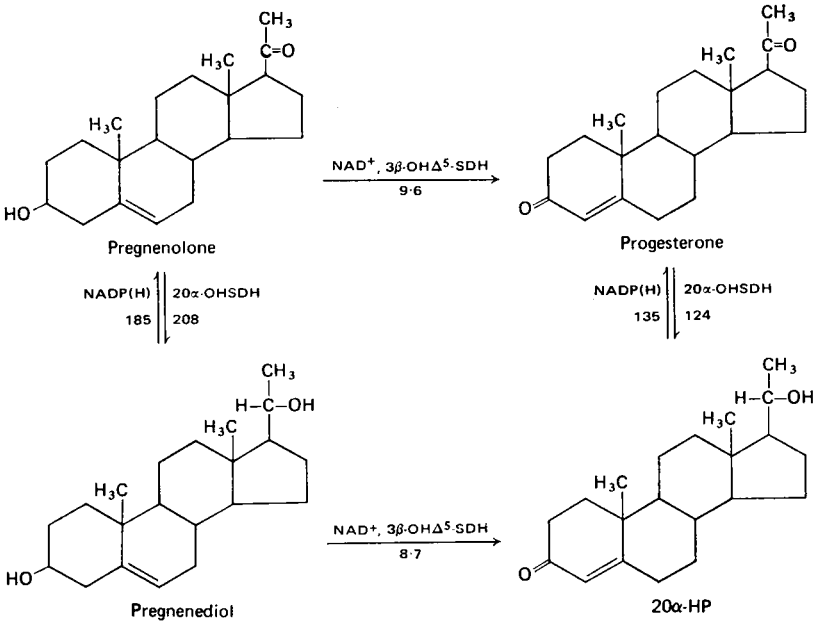
Fig. 2. Changes in steroid concentrations during parturition. (*a*) Ovarian progesterone (▽) and 20α-HP (▲). (*b*) 20α-HP/progesterone concentration ratios in the ovary (○) contrasted with those in peripheral plasma of the same rats (●) (data from Kuhn, 1969*a*).

contrasts with a simultaneous rise of the corresponding steroid ratio in peripheral (Fig. 2*b*) and ovarian venous plasma, and of the 20α-hydroxy steroid dehydrogenase activity in the ovary (Fig. 1).

Alternative metabolic routes from pregnenolone to 20α-HP

The observation (see below) that pregnenolone could replace progesterone in the 20α-hydroxy steroid dehydrogenase-catalysed oxidation of NADPH (eqn. 1) suggested the possibility of alternative routes from pregnenolone to 20α-HP (Scheme 1). In the route assumed by Wiest *et al.* (1968) pregnenolone is converted by 3β-hydroxy Δ^5-steroid dehydrogenase into progesterone, which is then reduced to 20α-HP by 20α-hydroxy steroid dehydrogenase. The new route reverses the order of these reactions and involves pregnenediol as an intermediate. Evidence follows for the demonstration of each individual reaction of Scheme 1 in cell-free and intact-cell systems.

Cell-free system. In optical assays, high-speed-supernatant fractions of homogenates prepared from ovaries after parturition catalysed the reduction of $NADP^+$ by either 20α-HP or pregnenediol, and the oxidation of NADPH by either progesterone or pregnenolone. Particulate fractions prepared from ovaries taken either before or after parturition



Scheme 1. Alternative biosynthetic pathways leading from pregnenolone to 20α-HP in ovarian homogenates. Numbers indicate enzyme activities (m-units/ovary at pH 7.4 at 37°C) in a single typical preparation of ovaries removed 2 days after parturition. Abbreviations: 3β-OHΔ5-SDH, 3β-hydroxy Δ5-steroid dehydrogenase; 20α-OHSDH, 20α-hydroxy steroid dehydrogenase.

catalysed the reduction of NAD^+ by either pregnenolone or pregnenediol, but not the reverse reactions. Typical activities determined optically at 37°C and pH 7.4 for ovaries removed 2 days after parturition are shown in Scheme 1.

In the particulate preparation $NADP^+$ was about 53% as effective as NAD^+, and pregn-5-ene-3β,20β-diol was about 84% as effective as pregn-5-ene-3β,20α-diol. In the high-speed-supernatant preparation pregn-5-ene-3β,20β-diol was not oxidized by either $NADP^+$ or NAD^+, and NAD^+ was unable to replace $NADP^+$ in the oxidation of pregnenediol.

Each reaction of Scheme 1 was also demonstrated by the use of radioactive precursor and characterization of the product by t.l.c. in the presence of authentic standard. Evidence is given in detail only for the formation and removal of pregnenediol, which constitutes the novel portion of the scheme. Thus the reduction of pregnenolone by NADPH in the presence of high-speed-supernatant gave a product that behaved like authentic pregnenediol on t.l.c. in solvents 1 and 2, and on paper in system 1. It gave a single radioactive peak on silver nitrate-impregnated thin-layer chromatograms in solvent 1 and on paper in system 2 (the positions of standards are difficult to locate in these systems), and after acetylation it migrated with authentic pregnenediol diacetate on t.l.c. in solvent 1. Incubation with intact corpora lutea gave 20α-HP (see below). Recrystallization with unlabelled carrier gave constant specific radioactivity (initial mixture, 9400; crystals, 7900, 7800, 7600, 8000 c.p.m./mg respectively). 20α-HP formed from pregnenediol + NAD^+ in the presence of particles, and pregnenolone formed from pregnenediol + $NADP^+$ in the presence of high-speed supernatant, also migrated with authentic carriers on t.l.c. in solvent 1.

Intact-cell system. Whole corpora lutea were taken from rats within 3h of parturition and incubated in Krebs improved Ringer 1 medium (Krebs, 1950) with appropriate ^{14}C- or ^3H-labelled precursor. Incorporation of the label into pregnenediol, 20α-HP, progesterone and pregnenolone was measured after their extraction and purification by chromatography. The following steroid conversions were shown in the standard incubation medium (Table 1).

(1) Pregnenolone incubated for 30min gave 20α-HP as the major product (37%) with smaller amounts of progesterone (9.5%) and pregnenediol (5.7%). Radioactive progesterone was converted into 20β-HP with crystalline 20β-hydroxy steroid dehydrogenase + NADH and shown to migrate with authentic standard on t.l.c. in solvent 1. The radioactive 20α-HP was mixed with unlabelled carrier and recrystallized to constant specific radioactivity (initial mixture, 6970; crystals, 6450, 6400, 6610, 6500 c.p.m./mg respectively). The pregnenediol migrated with authentic standard on t.l.c. in solvent 1 and on paper in system 1, and moved on paper with a single radioactive peak in system 2. Acetylation gave a single product migrating with authentic standard on t.l.c. in solvent 1. Recrystallization with unlabelled carrier gave constant specific radioactivity (initial mixture, 10900; crystals, 10200, 10400, 10300, 9800 c.p.m./mg respectively). Incubation with a particulate preparation of rat ovary and NAD^+ gave labelled

Table 1. *Steroid conversions by intact corpora lutea* in vitro

The standard incubation contained eight corpora lutea from a single rat, within 3h after parturition, in 1.0ml of Krebs improved Ringer 1 medium, containing pyruvate (Krebs, 1950), gassed with $O_2 + CO_2$ (95:5), at 37°C with frequent shaking. Modifications where indicated above were: L-lactate (replacing pyruvate at equal concentration); $N_2 + CO_2$ (95:5) (replacing $O_2 + CO_2$ in the gas phase); sodium azide (0.1M). Steroid precursors where indicated below were: [^3H]pregnenolone (1.6μg; 6.13×10^6 d.p.m.); [^3H]pregnenediol (2.2μg; 2.47×10^5 d.p.m.); [^{14}C]20α-HP (1μg; 4.12×10^4 d.p.m.); [^3H]progesterone (2μg; 3.74×10^5 d.p.m.). Steroids were extracted and purified as described in the Methods section.

Precursor	Incubation time (min)	Changes from standard incubation conditions	Products (% of precursor) 20α-HP	Progesterone	Pregnenediol	Pregnenolone
Pregnenolone	30	None	37	9.5	5.7	—
Pregnenolone	5	None	8.7	4.5	1.9	—
Pregnenolone	30	−Pyruvate, +lactate	53	4.3	5.7	—
Pregnenolone	5	−Pyruvate, +lactate	9.8	2.3	3.3	—
Pregnenolone	30	$-O_2, +N_2$	12	76	6.1	—
Pregnenolone	30	+Azide	15	15	2.1	—
Pregnenolone	30	−Glutamate	28	6.7	7.7	—
Pregnenediol	30	None	37	—	—	—
20α-HP	30	None	—	14	0.8	0.3*
Progesterone	30	None	31		2.2	1.1*

* Uncorrected for losses (usually about 30%) during purification.

20α-HP, characterized by t.l.c. in solvent 1 and by recrystallization with unlabelled carrier to constant specific radioactivity (initial mixture, 750; crystals, 735, 725, 765, 765 c.p.m./mg respectively).

(2) Pregnenediol incubated for 30 min gave 20α-HP in 37% yield. The product was mixed with unlabelled carrier and recrystallized to constant specific radioactivity (initial mixture, 4870; crystals, 4580, 4500, 4560, 4445 c.p.m./mg respectively).

(3) 20α-HP incubated for 30 min gave progesterone (13%), pregnenediol (0.8%) and pregnenolone (approx. 0.3%). This illustrates the reversibility of the 20α-hydroxy steroid dehydrogenase-catalysed reaction and the irreversibility of the 3β-hydroxy Δ^5-steroid dehydrogenase-catalysed reaction in intact cells.

(4) Progesterone incubated for 30 min gave 20α-HP (31%), pregnenediol (2.2%) and pregnenolone (approx. 1.1%).

Factors affecting the fate of pregnenolone

As a common precursor of progesterone and pregnenediol, pregnenolone stands at a branch point of metabolism. Scheme 1 illustrates how the direction of its metabolism may be determined by competition between the two hydroxy steroid dehydrogenases, and that pregnenediol may subsequently compete with pregnenolone for the common enzyme 3β-hydroxy Δ^5-steroid dehydrogenase.

Cell-free system. The direction of pregnenolone metabolism by crude homogenates at short incubation times was shown to depend on the relative activities of the two hydroxy steroid dehydrogenases. When the ovaries were taken before parturition the addition of $NAD^+ \pm NADPH$ resulted in the formation chiefly of progesterone. Unlabelled pregnenediol markedly inhibited this conversion (Table 2). When the ovaries were taken 2 days after parturition progesterone was again the main product in the presence of added NAD^+ only, but with the further addition of NADPH pregnenediol now accounted for 96% of the products and the formation of progesterone was inhibited (Table 2).

Direct substrate competition for 3β-hydroxy Δ^5-steroid dehydrogenase was also shown by measuring the formation of progesterone and 20α-HP from pregnenolone and pregnenediol respectively when these were added simultaneously (Table 3). The homogenate was prepared from ovaries taken before parturition to avoid 20α-hydroxy steroid dehydrogenase-catalysed interactions between these pathways. Progesterone and 20α-HP were formed in ratios approximately equal to those of their

Table 3. *Competition between pregnenediol and pregnenolone for the 3β-hydroxy Δ^5-steroid dehydrogenase of rat ovary homogenate*

Reaction mixtures contained homogenate from ovaries taken 4 days before parturition (equivalent to 0.25–4% of one ovary, the amount chosen so that substrate was not exhausted during the incubation), TES–KOH buffer, pH 7.4 (80 μmol), serum albumin (2 mg), NAD (0.3 μmol), [^{3}H]pregnenolone (0.5–10 μg, 7.97×10^4 d.p.m.) and [^{3}H]-pregnenediol (0.5–10 μg, 7.97×10^4 d.p.m.) in a final volume of 0.7 ml. Incubation was for 4 min at 37°C. Steroids were extracted and purified as described in the Methods section.

Added precursors (μg)		20α-HP/progesterone
Pregnenediol	Pregnenolone	product ratio
10	10	1.26
5	5	1.03
2	2	0.99
0.5	0.5	1.09
1	5	0.20
5	1	6.3

Table 2. *Pregnenolone metabolism to progesterone and pregnenediol by homogenates of rat ovary*

The complete system contained homogenate (equivalent to 2% of one ovary, approx. 1.5 mg), TES–KOH buffer, pH 7.4 (80 μmol), serum albumin (2 mg) and [^{3}H]pregnenolone (10 μg; 1.22×10^6 d.p.m.) in a final volume of 0.7 ml. NADPH (0.3 μmol) and pregnenediol were added as indicated. Reaction mixtures were incubated for 2 min at 37°C and steroids were extracted and purified as described in the Methods section.

Stage of ovarian development	Addition to complete system	Product formed (% of added pregnenolone)		
		Progesterone	20α-HP	Pregnenediol
4 Days before parturition	None	12.8	0.75	0.73
	NADPH	12.2	1.2	0.88
	None	13.9	—	—
	Pregnenediol (3 μg)	6.9	—	—
	Pregnenediol (6 μg)	4.7	—	—
	Pregnenediol (10 μg)	3.8	—	—
2 Days after parturition	None	4.2	0.43	1.2
	NADPH	1.1	2.5	24.4

added precursors, over a 25-fold range of ratio and a 20-fold range of net steroid concentration.

Intact-cell system (*Table* 1). In the standard incubation medium, more label deriving from pregnenolone is found after 30min in progesterone than in pregnenediol. Although much of the newly formed pregnenediol may have been further converted into 20α-HP, and although it was shown that added 20α-HP was partly converted into progesterone, nevertheless the results imply a relatively more rapid formation of progesterone than of pregnenediol. This was confirmed in 5min incubations, where secondary reactions were of less importance and where labelled progesterone accounted for one-third of the reaction products.

Replacement of pyruvate in the medium by lactate shifted the balance in favour of pregnenediol formation, so that progesterone accounted for only 18% and 7% of the products at 5 and 30min respectively. Pyruvate and lactate may affect the cytoplasmic $NAD^+/NADH$ concentration ratio through the action of lactate dehydrogenase (EC 1.1.1.27), whereas pyruvate could raise the cytoplasmic $NADP^+/NADPH$ concentration ratio by means of 'malic' enzyme (EC 1.1.1.40), which is active in this tissue (Flint & Denton, 1969). By altering the relative concentrations of NAD and NADPH, lactate and pyruvate could therefore alter the relative activities of the two hydroxy steroid dehydrogenases. Omission from the medium of glutamate, which might be converted into α-oxoglutarate and then into isocitrate with consequent drainage of NADPH, did not appreciably increase the labelling of pregnenediol. When terminal oxidation was inhibited by the presence of azide or by anaerobic conditions, in an attempt to promote the reduction of cellular $NADP^+$, progesterone was formed in unexpectedly large amounts with no increase in pregnenediol.

In these experiments the exchange of steroids between the medium and corpora lutea, as well as the interconversion of pregnenolone and pregnenediol on the one hand and progesterone and 20α-HP on the other, preclude the calculation of rates of pregnenolone metabolism along the two pathways depicted in Scheme 1. Nevertheless, in contrast with the situation in ovarian homogenates or in the intact animal, conversion into pregnenediol seems to be less rapid than to progesterone. This may reflect inadequacies of the incubation medium, or the low overall rate of 20α-HP formation (about 5% of the rate *in vivo*) due to permeability barriers.

Cell compartmentation of enzymes and their products

20α-Hydroxy steroid dehydrogenase occurs only in the soluble portion of ovarian homogenates (Wiest, 1959). In a homogenate of ovarian corpora lutea (49) in 0.25M-sucrose (12ml) fractionated by the method of Berthet & de Duve (1951) we found 3β-hydroxy Δ^5-steroid dehydrogenase mainly in the 'microsomal' fraction (94% of total activity) and glutamate dehydrogenase (EC 1.4.1.3) mainly in the 'mitochondrial' fraction (92% of total activity). The distribution of glutamate dehydrogenase indicates little mitochondrial damage, and hence little contamination of the 'microsomal' fraction by disrupted mitochondria. Therefore 20α-hydroxy steroid dehydrogenase and 3β-hydroxy Δ^5-steroid dehydrogenase appear to derive from the cytosol and the endoplasmic reticulum respectively. The latter enzyme is also largely microsomal in the adrenal gland (Beyer & Samuels, 1956).

Indirect information on the spatial separation of the enzyme products was obtained. When intact corpora lutea were incubated for 5min simultaneously with [^{3}H]progesterone and [^{14}C]pregnenediol, [^{3}H] and [^{14}C]20α-HP were formed by the actions of 20α-hydroxy steroid dehydrogenase and 3β-hydroxy Δ^5-steroid dehydrogenase respectively. The $^3H/^{14}C$ ratio of the 20α-HP purified from the medium (8.4) differed from that in the corpora lutea (2.1). Although steroid leakage into the medium probably bears no relation to secretion *in vivo*, this result implies that 20α-hydroxy steroid dehydrogenase and 3β-hydroxy Δ^5-steroid dehydrogenase release their products into separate cell compartments. If so, pregnenediol and progesterone are also normally released into separate compartments that, from the intracellular location of these enzymes, may be associated with the cytosol and the endoplasmic reticulum respectively.

DISCUSSION

This investigation concerns the change from progesterone to 20α-HP as the major steroid secreted by rat ovarian corpora lutea at the end of pregnancy.

Changes in ovarian enzymes and steroids. The rapid increase in activity of 20α-hydroxy steroid dehydrogenase just before parturition (Fig. 1) (Wiest *et al.* 1968) coincides with the increased 20α-HP/progesterone concentration ratio in ovarian venous (Fajer & Barraclough, 1967; Hashimoto *et al.* 1968) and peripheral (Wiest *et al.* 1968; Kuhn, 1969*a*) plasma. This enzyme is located in the corpora lutea (Wiest *et al.* 1968), and its slow decay during lactation parallels the morphological decay of the corpora lutea of pregnancy (Long & Evans, 1922). However, it contributes little to the type of steroid secreted after day 2 of lactation (Tomogane *et al.* 1969), when the newly formed corpora lutea of lactation take over as the main site of steroidogenesis. Its measured activity just after parturition is about 50-fold greater than the rate of 20α-HP secretion, which is itself greatest at this time.

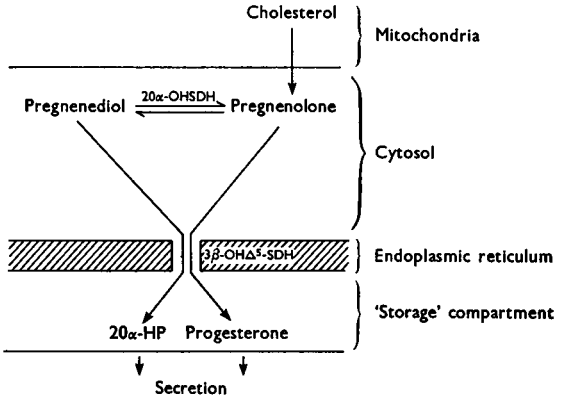
Clearly this enzyme plays a major role in the change from progesterone to 20α-HP secretion at parturition, and a similar role has been ascribed to it during the oestrous cycle (Wiest, 1959).

The activity changes of 3β-hydroxy Δ^5-steroid dehydrogenase (Fig. 1), which catalyses the formation of progesterone from pregnenolone, do not correlate with changes in either the amount or type of steroid that is secreted. However, just after parturition, when its measured activity is lowest and steroid secretion is greatest, it is present in five-fold excess of metabolic requirements.

Progesterone and 20α-HP in ovarian tissue (Fig. 2) show changes in amount strikingly different from those so far discussed, although they are assumed to give rise to the same steroids in the plasma. In particular, the 20α-HP/progesterone concentration ratio falls steeply on the last day of pregnancy, when the activity of 20α-hydroxy steroid dehydrogenase rises most rapidly. Such a change is away from the equilibrium ratio that would be set up according to eqn. (1) and which is calculated to be about 48 (see below). This is difficult to reconcile with their proposed interconversion by this enzyme, and suggests that the measured progesterone and 20α-HP are not in contact with 20α-hydroxy steroid dehydrogenase but represent material in transit before secretion. This conclusion is consistent with the above results on cell compartmentation of enzymes and their products.

Separate pathways for the synthesis of progesterone and 20α-HP: the roles of 20α-hydroxy steroid dehydrogenase and pregnenediol on a progesterone bypass. In the light of the above conclusions the existence of a separate pathway for the formation of 20α-HP from pregnenolone assumes importance. We have shown in cell-free and intact-cell systems that pregnenolone is converted by 20α-hydroxy steroid dehydrogenase into pregnenediol and thence by 3β-hydroxy Δ^5-steroid dehydrogenase into 20α-HP, presumably via the intermediate 20α-hydroxypregn-5-en-3-one. The capacity of each reaction exceeds the physiological rate of 20α-HP secretion. Preliminary evidence for the presence of pregnenediol in rat ovaries has been obtained by Dr M. Holzbauer (personal communication).

Pregnenediol occurs normally in the plasma (Wilson, Mortimer, Lipsett & Ryan, 1961: Sjövall & Vihko, 1966) and umbilical cord blood (Eberlein, 1965) of humans, and in the urine of pregnant mares (Marker & Rohrmann, 1938). It is found in the urine of some patients with adrenal tumours (Hirschmann & Hirschmann, 1945; Schiller, Miller, Dorfman, Sevringhaus & McCullagh, 1945) or abnormal ovaries (Schiller *et al.* 1945) or suffering from congenital adrenal hyperplasia (Bongiovanni, 1961). It is formed from added pregnenolone by cultured adrenal tumour cells (Pierson, 1967; Kowald & Fiedler, 1968). To our knowledge no role for pregnenediol has been proposed in ovarian metabolism.



Scheme 2. Proposed pathways leading *in vivo* from pregnenolone to progesterone and 20α-HP. The scheme shows the site of action of 20α-hydroxy steroid dehydrogenase and the role of pregnenediol as a precursor of 20α-HP. The locations of enzymes and steroids within the cell are depicted. The mitochondrial origin of pregnenolone is assumed on the basis of studies in bovine corpora lutea (Hall & Koritz, 1964). Abbreviations: 3β-OHΔ^5-SDH, 3β-hydroxy Δ^5-steroid dehydrogenase; 20α-OHSDH, 20α-hydroxy steroid dehydrogenase.

In other tissues that synthesize substances for secretion the terminal reaction is catalysed by a particulate enzyme associated with the endoplasmic reticulum. If this applies to 20α-HP, as it does to progesterone, then only the pathway involving 3β-hydroxy Δ^5-steroid dehydrogenase as the last enzyme can be operative. This consideration, together with the apparent spatial separation of 20α-hydroxy steroid dehydrogenase from progesterone and 20α-HP within the cell, suggests that 3β-hydroxy Δ^5-steroid dehydrogenase may synthesize these steroids vectorially into a 'storage compartment', possibly associated with the tubules of the endoplasmic reticulum or the Golgi apparatus. Scheme 2 illustrates the conclusions reached, and forms the basis for discussion below. Attention is drawn to the fact that Scheme 2 is not simply applicable to the metabolism of pregnenolone by corpora lutea *in vitro*, where the movement of newly synthesized progesterone and 20α-HP via the medium into the cytosol brings them into contact with 20α-hydroxy steroid dehydrogenase.

Regulation of synthesis of progesterone and 20α-HP. If it is assumed that 20α-HP and progesterone are not significantly metabolized to other substances in the ovary, at steady state their relative rates of secretion are equal to their relative rates of formation by 3β-hydroxy Δ^5-steroid dehydrogenase. These are determined by the

pregnenediol/pregnenolone concentration ratio in the cytoplasm (see Table 3), which in turn reflects the activity of 20α-hydroxy steroid dehydrogenase relative to that of 3β-hydroxy Δ^5-steroid dehydrogenase (Table 2). Whether the finally secreted steroid is the physiologically active progesterone or the inactive 20α-HP therefore hinges on the competition between these enzymes for pregnenolone.

During pregnancy 20α-HP and progesterone are secreted in a ratio approx. 0.6 (Hashimoto *et al.* 1968). Since the measured 20α-hydroxy steroid dehydrogenase/3β-hydroxy Δ^5-steroid dehydrogenase activity ratio is not greater than 0.1 at this time (Fig. 1), factors other than the total amount of enzyme must determine their activities *in vivo*. Concentrations of NAD^+ and NADPH, the different cellular locations of these enzymes and their relative affinities for pregnenolone may be responsible.

From 1–2 days before parturition the increased activity of 20α-hydroxy steroid dehydrogenase relative to that of 3β-hydroxy Δ^5-steroid dehydrogenase diverts pregnenolone towards the formation of pregnenediol and thence to 20α-HP, decreasing the formation of progesterone through both a decrease in concentration of pregnenolone and the formation of pregnenediol, which competes with pregnenolone for 3β-hydroxy Δ^5-steroid dehydrogenase. Shortly after parturition, when 20α-hydroxy steroid dehydrogenase reaches a maximum activity that is about tenfold greater than that of 3β-hydroxy Δ^5-steroid dehydrogenase and about 50-fold greater than the secretion rate of progesterone and 20α-HP, one might expect pregnenolone and pregnenediol to be in equilibrium with the free $NADP^+$ and NADPH of the cytoplasm according to the equation:

$$\text{Pregnenolone} + \text{NADPH} + H^+ \rightleftharpoons \text{pregnenediol} + NADP^+ \quad (2)$$

The free $NADP^+$/NADPH concentration ratio in normal rat liver is 0.0143 (Krebs & Veech, 1969), and is also likely to be low in other tissues performing reductive syntheses. Wiest & Wilcox (1961) have determined the equilibrium constant K to be 1.70×10^7 for the interconversion of progesterone and 20α-HP by 20α-hydroxy steroid dehydrogenase (eqn. 1). From these values the calculated pregnenediol/pregnenolone equilibrium concentration is 48 (pH 7.4)–119 (pH 7.0). The highest recorded 20α-HP/progesterone concentration ratio is 53, in ovarian venous plasma immediately after parturition (Hashimoto *et al.* 1968). Therefore under conditions of high 20α-hydroxy steroid dehydrogenase activity the secreted 20α-HP/secreted progesterone ratio approaches a value limited by the free $NADP^+$/NADPH concentration ratio of the cytoplasm.

In conclusion, the sharp fall in progesterone secretion that precedes parturition and lactogenesis is due to a combination of two events: first, a decrease in the net steroidogenic activity of the ovary; secondly, a re-direction of pregnenolone from one pathway leading to progesterone to another, distinct, pathway leading to 20α-HP.

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