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Progesterone-synthesizing ability of preovulatory follicles of cows relative to the peak of LH

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Summary. Preovulatory cow follicles ($n = 34$) were collected at different times after the onset of oestrus until shortly before ovulation. In-vitro conversion of tritiated pregnenolone in the presence of NAD^+ by homogenates of the follicular wall was compared in phases relative to the LH peak. During phase 0 (before the LH surge) a moderate conversion into progesterone occurred, but it was subsidiary to that into 17α-hydroxypregnenolone and other unidentified steroids. During phases 1 (0–6 h after the LH peak), 2A (6–14 h) and 2B (14–20 h) the production of progesterone and 17α-hydroxypregnenolone remained constant; at phase 2B the percentage of remaining pregnenolone was higher than in the preceding phases. In phase 3 (20 h after the LH peak until ovulation) conversion into progesterone had increased about 4-fold to the highest levels observed (97% after 2 h incubation), and production of 17α-hydroxypregnenolone and unidentified steroids was low. In an additional experiment, homogenates of the wall of 3 follicles at phase 3 were also incubated with tritated progesterone in the presence of NADPH. The percentage of remaining progesterone was high, and a moderate conversion into 17α-hydroxyprogesterone occurred. In the main experiments, however, production of this steroid was not observed.

The results indicate that steroid synthesis in the preovulatory follicle of the cow changes to the production of progesterone shortly before ovulation.

Introduction

After ovulation, differentiation of the cells of the follicular wall into luteal cells, which constitute the corpus luteum producing progesterone, appears to be initiated by luteinizing hormone (LH) (see Fritz & Speroff, 1982, for review). In several mammalian species a sometimes transitory increase of the progesterone concentration in the follicular fluid is observed before ovulation (man: McNatty, Hunter, McNeilly & Sawers, 1975; rat: Szoltys, 1976: pig: Ainsworth, Tsang, Downey, Marcus & Armstrong, 1980; sheep: Murdoch & Dunn, 1982), which is considered to be a sign of the onset of functional luteinization. In the cow, a sharp increase of the progesterone concentration in the fluid of preovulatory follicles takes place shortly before ovulation during phase 3 of the preovulatory development (Dieleman, Kruip, Fontijne, de Jong & van der Weyden, 1983b). A preliminary experiment (S. J. Dieleman & Th. A. M. Kruip, unpublished observations) indicated a rapid conversion of pregnenolone into progesterone in the presence of NAD^+ shortly before ovulation.

In the present study, therefore, we investigated whether the progesterone-synthesizing ability of preovulatory cow follicles increases at about 20 h after the preovulatory peak of LH. Also, possible conversion of progesterone was examined shortly before ovulation to determine whether progesterone is an end-product at this stage of follicular development.

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Materials and Methods

Collection of follicles. Preovulatory follicles from Dutch-Friesian heifers ($n = 34$) with normal cycles were collected at known times after the onset of oestrus until ovulation (Dieleman *et al.*, 1983b; Dieleman, Bevers, Poortman & van Tol, 1983a). Blood samples for the radioimmunoassay (RIA) of LH were collected at hourly intervals from the time of luteal regression, as determined by estimating the progesterone concentration in the peripheral blood, twice a day with a rapid RIA, until at least 24 h after onset of oestrus. Immediately after recovery of the ovaries by ovariectomy the largest follicle, being preovulatory (Dufour, Whitmore, Ginther & Casida, 1972), was dissected free of stromal tissue and its diameter was measured under a stereomicroscope as described by Kruip & Dieleman (1982). On the basis of histological examination of a small piece of the follicular wall, the follicles used were identified as non-atretic (Dieleman *et al.*, 1983b).

Chemicals. When not specified, chemicals were of analytical reagent grade and obtained from Merck AG, Darmstadt, West Germany; organic solvents were distilled just before use. [7(n)-^{3}H]Pregnenolone (sp. act. 0·370 PBq/mol; tritium distribution over positions 7α, 7β and 4: 52, 28 and 20% respectively) and [1,2,6,7(n)-^{3}H] progesterone (sp. act. 3·22 PBq/mol) were purified by thin-layer chromatography (t.l.c.; DC Fertigplatten F 254; Merck AG). These tracers and [4-^{14}C]pregnenolone (sp. act. 2·07 TBq/mol) and [4-^{14}C]progesterone (sp. act. 2·07 TBq/mol) were obtained from Amersham International Ltd, Bucks, U.K., and (4-^{14}C]17α-hydroxyprogesterone (sp. act. 1·85 TBq/mol) from New England Nuclear, Boston, MA, U.S.A.; the ^{14}C-tracers were subjected to routine checks for purity by t.l.c. and, if necessary, purified by filtration on Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Steroids were purchased from Steraloids Inc. (Wilton, NH, U.S.A.). Aqueous samples for radioactivity measurements were dispersed in a xylene-based mixture and counted as described previously (Dieleman *et al.*, 1983b). Samples in organic solvents were dissolved in Instafluor II (Packard Instruments Company, Downers Grove, IL, U.S.A.) and counted as described previously (Dieleman & Blankenstein, 1984).

Incubation. After dissecting the preovulatory follicles, the follicular wall was weighed and subsequently homogenized at 0°C in 13 ml Tris–HCl buffer (0·01 M, pH 7·4) with mannitol (0·25 M). The mean interval from ovariectomy until homogenization was 31·8 ± 13·4 (s.d.) min ($n = 34$). The homogenates were centrifuged at 900 *g* for 20 min at 4°C and the supernatant was used for incubation. The incubation mixture consisted of 0·5 ml propyleneglycol containing about 0·158 MBq [7(n)-^{3}H]pregnenolone and 3 ml supernatant, to which was added 0·5 ml of a solution of NAD^{+} (Boehringer, Mannheim, West Germany) in Tris–HCl–mannitol buffer (final concentration of NAD^{+} 0·2 mM). The incubations were carried out under continuous shaking at 39°C (body temperature of the cow) in an air atmosphere. After 15, 30, 60 and 120 min, 0·9 ml samples were pipetted from the incubation mixture and the enzyme reactions were stopped by the addition of 3 ml dichloromethane; thereafter the fractions were stored at −25°C. In an additional experiment a further 3 ml of the supernatant ($n = 3$) were incubated similarly for 120 min with about 0·343 MBq [1,2,6,7(n)-^{3}H]progesterone in the presence of NADPH (Boehringer; final concentration of NADPH 0·2 mM). Blank incubations with 3 ml Tris–HCl–mannitol buffer instead of homogenate showed no conversion of pregnenolone after 15, 30, 60 and 120 min incubation ($n = 4$) respectively.

Extraction and processing for incubations with tritiated pregnenolone. After 4-fold extraction each time with 6 ml dichloromethane (recovery: 98·5 ± 0·5%, $n = 136$), a mixture of ^{14}C-labelled pregnenolone and progesterone (about 1·0 and 1·1 kBq respectively) was added in a known proportion in 10 µl for procedural recovery measurement and recrystallization purposes. Subsequently, the residues after evaporation *in vacuo* were subjected to t.l.c. The plates were developed 3 times in benzene:cyclohexane (1:1, v/v; System 1) to remove fatty materials, and twice in chloroform: methanol (99:1, v/v; System 2). Radioactive areas were recorded on radioscans using a thin-layer scanner (LB 2723/6280/241 K; Berthold-Frieseke, Karlsruhe, West Germany) with a sensitivity

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which permitted detection of conversion percentages of steroid $>1\%$. Thereafter the fractions containing pregnenolone and progesterone were further purified by recrystallization up to a constant ratio of $^3H/^{14}C$ activities, and the area corresponding with 17α-hydroxypregnenolone was isolated. For 5 fractions, 17α-hydroxypregnenolone was further purified by t.l.c. (System 2) and subsequent recrystallization to constant specific activity. The conversion percentage of progesterone and the percentage of pregnenolone remaining were calculated from the ^3H activity of the pure steroids in proportion to the total ^3H activity after extraction and corrected for recovery. That of 17α-hydroxypregnenolone was estimated by correcting the ^3H activity of the corresponding fraction after t.l.c. (Systems 1 and 2) for the mean recovery ($75 \cdot 8 \pm 10 \cdot 2\%$, $n = 8$). The coefficient of variation due to statistical errors in measurements of radioactivity was <4 and $<15\%$ for percentage conversions of >1 and $<0 \cdot 5$ respectively.

Extraction and processing for incubations with tritiated progesterone. The extraction and purification procedures were similar to those mentioned above. The t.l.c. fraction containing 17α-hydroxyprogesterone was subjected again to t.l.c. (benzene:ether, 4:1, v/v; developed twice), after which 17α-hydroxyprogesterone was further purified by recrystallization to a constant ratio of $^3H/^{14}C$ activities.

Statistical analysis. The paired t test (two-tailed) was used to compare the means of two samples. Differences between the means of samples in grouped data were tested for significance by analysis of variance according to Scheffé (1959).

Results

The time when the maximum LH concentration was recorded in peripheral blood was defined as the LH peak. The mean ($\pm$ s.d.) intervals between onset of oestrus, i.e. first standing oestrus, and the onset of the LH surge (first elevated concentration of LH followed by higher values) and between the maximum LH value and ovulation, have been reported previously to be $1 \cdot 7 \pm 2 \cdot 4$ and $24 \pm 1 \cdot 4$ h respectively (Dieleman *et al.*, 1983b).

Before the LH surge (phase 0), 6 follicles were collected; the mean ($\pm$ s.e.m.) diameter and weight of the follicular wall were $16 \cdot 2 \pm 0 \cdot 8$ mm and 209 ± 27 mg respectively. After the LH peak 27 follicles were collected: 6 at 0–6 h after the LH peak (phase 1; size and weight: $16 \cdot 0 \pm 1 \cdot 0$ mm and 227 ± 39 mg respectively), 6 at 6–14 h (phase 2A; $15 \cdot 7 \pm 0 \cdot 5$ mm and 268 ± 44 mg), 5 at 14–20 h (phase 2B; $18 \cdot 1 \pm 0 \cdot 6$ mm and 335 ± 33 mg) and 10 between 20 h and ovulation (phase 3; $16 \cdot 9 \pm 0 \cdot 7$ mm and 317 ± 36 mg). In one heifer (16 mm, 209 mg) ovulation had taken place shortly before ovariectomy, and the interval after the LH peak was 23 h. The diameter of the follicles varied from 13·7 to 20 mm independently of the stage of preovulatory development. Analysis of variance proved the apparent increase in weight of the follicular wall to be non-significant.

Table 1. Conversion percentage of 17α-hydroxypregnenolone and the percentage of unidentified steroid after 120 min incubation with [7(n)-^3H]pregnenolone of homogenates of preovulatory cow follicles in the phases between onset of oestrus and ovulation

Phase	Period* (h)	No. of follicles	17α-Hydroxy-pregnenolone	Unidentified steroid
0	Before	6	38·5±3·3	33·5± 3·5
1	0–6	6	43·1±6·7	28·8± 7·5
2A	6–14	6	51·1±4·9	28·1± 5·5
2B	14–20	5	40·3±7·2	21·5±10·9
3	20–25	10	10·9±3·7	8·4± 2·4

Values are mean ± s.e.m.
* In relation to LH peak level.

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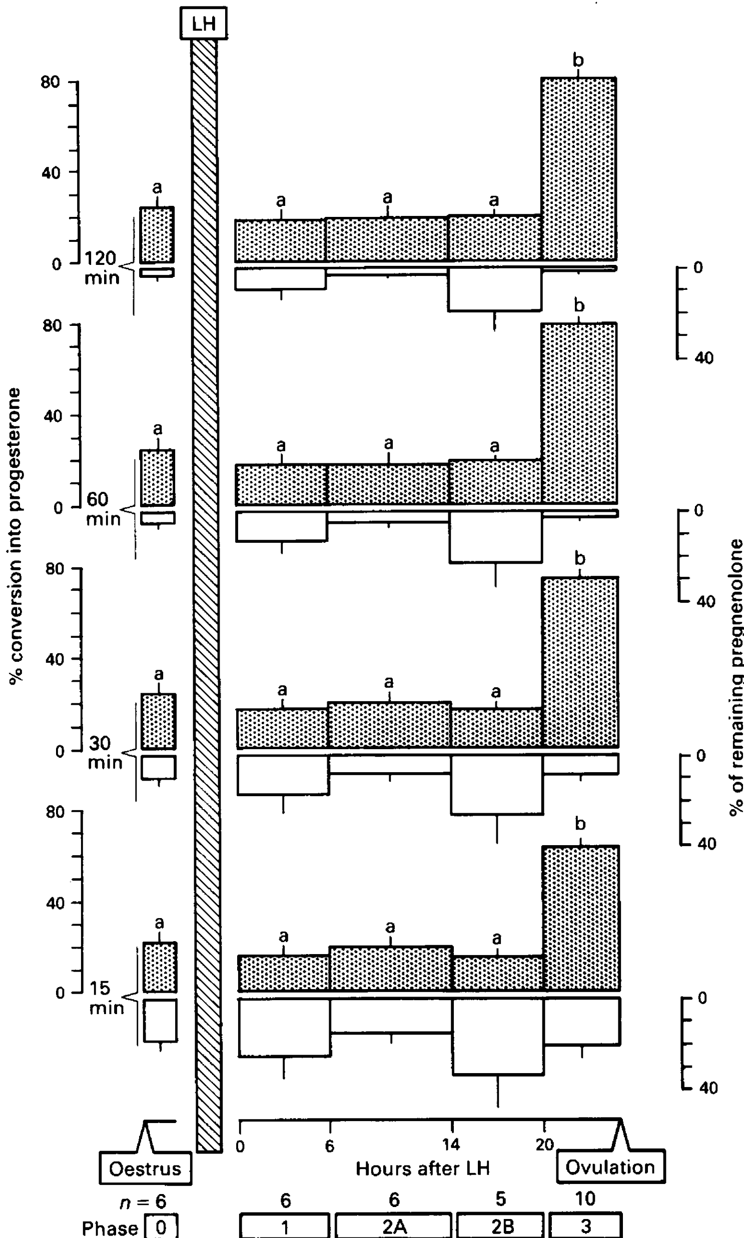

Text-fig. 1. Conversion percentage of progesterone (stippled histograms) and the percentage of remaining pregnenolone (open histograms) after incubation for 15, 30, 60 and 120 min with [7(n)-^{3}H]pregnenolone of homogenates of preovulatory cow follicles in the phases between onset of oestrus and ovulation. Values are mean ± s.e.m. for the no. of follicles indicated (*n*). At each incubation time and hormone, values with a different superscript letter are significantly different ($P < 0.005$).

Conversion of [7(n)-^{3}H]pregnenolone into progesterone and intermediary steroids

Text-figure 1 shows the mean conversion percentage of progesterone and the percentage of pregnenolone remaining after incubation of homogenates of preovulatory bovine follicles in the

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phases between the onset of oestrus and ovulation with the results of the statistical analysis. The production of progesterone showed a significant ($P < 0.005$) increase during incubation at phase 3. The decrease during incubation of the percentage of pregnenolone remaining was significant ($P < 0.05$) at all phases, except at phase 2B. Follicles at phases 0, 1, 2A and 2B showed an appreciable production of 17α-hydroxypregnenolone (Table 1). The unidentified steroid fraction (Table 1) was distributed over several areas on t.l.c.; no peaks were detected on the radioscans concurring with reference 17α-hydroxyprogesterone. At phase 3, when conversion into progesterone had increased sharply (Text-fig. 1), conversion into 17α-hydroxypregnenolone was significantly ($P < 0.01$) less than in the preceding phases. Two follicles which showed a high conversion percentage of progesterone (97·6 and 96·6% after 120 min incubation, respectively) did not produce detectable amounts of 17α-hydroxypregnenolone. The one follicle collected shortly after ovulation showed a high conversion percentage of progesterone after 15, 30, 60 and 120 min incubation (72·7, 87·7, 94·7 and 96·9% respectively), and the percentages of pregnenolone remaining were 25·6, 10·4, 2·6 and 0·6% respectively; the conversion percentage of 17α-hydroxypregnenolone after 120 min incubation was 2·46%.

Conversion of [1,2,6,7(n)-^{3}H]progesterone

The conversion percentage of 17α-hydroxyprogesterone and the percentage of progesterone remaining, after 120 min incubation with [1,2,6,7(n)-^{3}H]progesterone of homogenates of preovulatory bovine follicles at phase 3, were 22·4 ± 2·4 and 75·6 ± 1·5% ($n = 3$) respectively.

Discussion

Changes in progesterone-synthesizing ability of preovulatory bovine follicles relative to the preovulatory peak of LH were investigated in an in-vitro system using homogenates of the follicular wall, with tritiated pregnenolone as precursor. Although in this way the in-vivo intercellular contact and intracellular organization are lost and no data are available about endogenous pregnenolone concentrations and production, it is assumed that this system reflected in-vivo changes in progesterone-synthesizing ability during preovulatory follicular development.

Before the LH surge preovulatory bovine follicles were able to produce progesterone, but high conversion percentages were observed for 17α-hydroxypregnenolone and for the unidentified steroid fraction, which may have consisted of further intermediates of the Δ5-pathway. This is consistent with the preference for the Δ5-pathway in cow follicles as reported by Lacroix, Eechaute & Leusen (1974). It is suggested that at phase 0 steroid synthesis of the preovulatory follicle is directed mainly to the production of oestradiol, as is reflected by the considerable oestradiol concentration in the fluid of follicles at the time, which is about 15-fold higher than that of progesterone (Dieleman *et al.*, 1983b). During phases 1, 2A and 2B the moderate ability to produce progesterone was continued, and conversion of pregnenolone into the other steroids apparently prevailed. At phase 3, from 20 h after the LH peak until ovulation, however, a steep 4-fold increase of the conversion, into progesterone occurred. In 2 out of the 10 follicles collected at this phase pregnenolone was converted almost completely into progesterone alone; the one follicle collected shortly after ovulation showed a similar pattern. At phase 3 progesterone is probably the end-product, since conversion of this steroid into other steroids was limited in the presence of NADPH. Moreover, no conversion into 17α-hydroxyprogesterone could be detected (i.e. conversion < 1%) throughout preovulatory development. The high progesterone-synthesizing ability at phase 3 coincides with the previously reported 5-fold increase of the progesterone concentration in the follicular fluid (Dieleman *et al.*, 1983b). This indicates that, in the cow, the high progesterone concentration in preovulatory follicles shortly before ovulation is not merely due to accumulation of progesterone into the fluid as supposed in the sheep (Murdoch & Dunn, 1982), but to an increase of progesterone synthesis. In preovulatory follicles of sheep the progesterone concentration increases near the time

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of ovulation, but the increase is not reflected by the progesterone concentration in the ovarian venous blood (Murdoch & Dunn, 1982). These authors speculated that this might be due to a reduced efflux of progesterone from the follicular fluid as a result of prostaglandin F-2α acting as a vasoconstrictor.

The coincidence of the sharp increase in progesterone-synthesizing ability with the previously observed morphological luteinization of the granulosa cells (Dieleman *et al.*, 1983b) suggests that the membrana granulosa is the major source of progesterone in preovulatory cow follicles at this stage of follicular development, as in the pig (Evans, Dobias, King & Armstrong, 1981). The moderate conversion into progesterone during the phases before until 20 h after the LH peak possibly has to be attributed to the theca interna (Fortune & Hansel, 1979a). The temporary increase of the progesterone concentration in the fluid of follicles at phase 1 (0–6 h after the LH peak; Dieleman *et al.*, 1983b), however, was not reflected in the present in-vitro system. The lag between the termination of granulosa aromatizing ability at 14 h after the LH peak (Dieleman & Blankenstein, 1984) and the onset of high progesterone production at 20 h may indicate that the Δ5-3β-hydroxysteroid dehydrogenase, necessary to convert pregnenolone into progesterone, is newly synthesized or activated upon differentiation of the membrana granulosa into lutein cells. Contrary to this, the increase of progesterone formation in rat preovulatory follicles appears to be due to inhibition of 17α-hydroxylase activity (Lieberman *et al.*, 1975).

Explanted granulosa cells of cow follicles luteinize in about 2 days in culture, losing their aromatizing ability (Henderson & Moon, 1979), and addition of oestradiol in a concentration similar to that observed in preovulatory cow follicles at oestrus prevents in-vitro luteinization (Fortune & Hansel, 1979b). Despite this, it is supposed that luteinization *in vivo* in preovulatory cow follicles is caused by the preovulatory surge of LH, since this process became apparent at about 20 h after the LH peak and about 14 h after the onset of the decrease of the oestradiol concentration in the fluid (Dieleman *et al.*, 1983b), as opposed to the 2 days *in vitro*. It is not known whether the high concentration of follicle-stimulating hormone (FSH) observed in the fluid of cow follicles at phase 3 (Dieleman *et al.*, 1983a) is also required to stimulate progesterone synthesis; in the rat (Wang & Chan, 1982), FSH stimulates granulosa progesterone production *in vitro*. The function of the high progesterone production at the time is not well understood, but it may be involved in oocyte maturation (Kruip, Cran, Van Beneden & Dieleman, 1983) and ovulatory processes (see Peters & McNatty, 1980, for review).

The present results indicate that the preovulatory LH surge changes the preovulatory cow follicle from an oestradiol-producing gland at oestrus to one in which progesterone synthesis is dominant shortly before ovulation.

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