Progesterone and Δ^4 Pregnen-20β-ol-3-one in Bovine Reproductive Organs and Body Fluids.* (27099)

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It is well established that progesterone is required in preparation of the uterus for implantation as well as for maintenance of early pregnancy. As a result of this physiologic significance, investigations have been conducted on the occurrence of this hormone and related substances in various tissues and body fluids of different species including the cow. *In vitro* synthesis of progesterone by bovine corpora lutea has been stimulated with ovine LH, HCG and equine pituitary gonadotrophin(1). Natural occurrence of a second progestin, Δ^4 pregnen-20β-ol-3-one (20β-ol), was first observed in bovine luteal tissue by Gorski *et al.*(2). *In vitro* studies have also demonstrated the ability of these organs to reduce progesterone to 20β-ol(3). In this investigation concentrations of progesterone and 20β-ol were determined in bovine corpora lutea, placenta and associated fluids and blood plasma at selected stages of the estrous cycle and pregnancy. Changes in progestin concentrations were also determined after luteal tissue was allowed to stand under various conditions. In addition the capacity of luteal tissue to synthesize these progestins *in vitro* was investigated.

Materials and methods. Four corpora lutea were taken from each of 3 Hereford heifers, 2 at day 8 and 2 at day 16 of the estrous cycle. Flank incisions were made under local anesthesia to enucleate the corpora lutea. One or more estrous cycles were observed before subsequent corpora lutea were removed. After enucleation, the glands were sliced, combined and representative samples were treated as follows: (1) placed in 95% ethanol within 5 min. after removal from the animal; (2) maintained at 37°C for 30 min. in a sealed jar moistened with Krebs Ringer bicarbonate glucose medium (pH 7.4); (3) same as 2 but allowed to stand 8 hr at 27°C; (4) incubated for 2 hr in Krebs Ringer bicarbonate glucose medium as described by Duncan *et al.*(4).

Corpora lutea from pregnant animals of mixed breeding were obtained at time of slaughter. Duration of pregnancy was estimated from crown rump measurements of fetuses(5). These glands were excised 30 to 45 min. after slaughter and halved for duplicate determinations. Tissues were weighed, placed in vials containing 95% ethanol and stored at –15°C. Placental tissues and fluids obtained from some of these animals were weighed and frozen for subsequent analysis. Blood samples were obtained from the jugular vein of beef cows (Hereford, Angus and Shorthorn) at known stages of pregnancy.

Following the various experimental treatments, luteal tissue samples were homogenized, extracted with 95% ethanol and subjected to chromatography on aluminum oxide columns as described by Duncan *et al.*(4). Fractions containing α, β unsaturated steroids were evaporated to dryness under reduced pressure at 60°C. Residues were transferred to 125 ml separatory funnels with 3-10 ml portions of petroleum ether followed by 2-5 ml portions of 70% methanol and were partitioned between the solvents. Methanolic solutions were transferred to a second separatory funnel containing 30 ml of petroleum ether. After partitioning in the second funnel, methanolic solutions were transferred into 300 ml flasks and 7 additional 10 ml portions of 70% methanol were partitioned with the petroleum ether solutions in the separatory funnels. Pooled methanolic solutions,

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which theoretically contained more than 99% of the 20β-ol and 98.5% of the progesterone introduced in the first funnel, were subsequently subjected to paper chromatography. Placental tissues were finely ground, digested with 5% NaOH and extracted with ether as described by Noall *et al.*(6). Ketonic and non-ketonic fractions were separated with Girards T reagent by the method of Talbot *et al.*(7). Ketonic fractions were further purified with solvent partitioning as described for the luteal tissue followed by paper chromatography. Blood plasma and placental fluids were processed by the method of Short (8).

Descending paper chromatograms were developed with a Skellysolve C-80% methanol solvent system(9). Areas of the chromatogram containing the progestins were located by reference chromatograms and scanning with ultra-violet light. After elution steroids were quantitated from observed absorbancies at 230, 240 and 250 mμ using the equation of Allen(10) and standard curves prepared from 95% ethanolic solutions of progesterone and Δ^4 pregnen-20β-ol-3-one.†

In recovery experiments, 95% ethanol solutions containing 5 to 50 μg of the progestins were added to aliquots of homogenized or ground tissues, blood plasma and placental fluids. Mean recovery values in 12 experiments with luteal tissue were 74.7% for progesterone and 76.6% for 20β-ol. One recovery experiment with placental tissue gave values of 80.8 and 55.8% for progesterone and 20β-ol respectively. An average of 63.0% of progesterone added to 2 plasma aliquots was recovered. Two recovery experiments with placental fluids resulted in values of 85.7% for progesterone and 84.2% for 20β-ol. All data in this investigation were adjusted with these recovery values.

Results and discussion. Little difference was observed in mean weight of corpora lutea from heifers at days 8 and 16 of the estrous cycle, 4.39 and 4.57 g respectively. At day 8 endogenous progesterone averaged 29.2 μg g and 20β-ol averaged 0.3 μg g of luteal tissue. Mean endogenous concentrations of progesterone and 20β-ol were both greater at day 16, 47.2 and 2.0 μg/g respectively. Following 2 hr incubation mean progesterone concentration was 75.4 μg/g in luteal tissue from day 8 and 100.6 μg/g at day 16. Mean concentration of 20β-ol following incubation was 2.4 μg/g at day 8 and 14.4 μg/g at day 16. Increases in progesterone concentration during incubation were nearly the same at both days, 46.2 and 53.4 μg/g at days 8 and 16 respectively. These results are similar to those obtained previously with swine luteal tissue in which initial levels of progesterone were also lower at day 8 than at 16 and increases in concentration during 2 hr incubation were also nearly the same, 73 and 72 μg/g respectively, at the 2 stages(4). Endogenous concentrations of the progestins are in general agreement with those reported for other chemical assays(11,12).

Luteal tissue allowed to stand for 30 min. at 37°C following removal from animals had mean progesterone and 20β-ol concentrations of 34.1 and 1.0 μg/g respectively at day 8 and 53.1 and 5.2 μg/g at day 16. Only progesterone concentration at day 16 differed significantly from endogenous levels ($P < 0.05$). This may indicate that progesterone concentrations determined in corpora lutea obtained at slaughter are higher than endogenous levels at this stage and possibly during pregnancy. Mean concentrations of 34.6 and 4.1 μg/g were observed for progesterone and 20β-ol respectively after luteal tissue from day 8 had stood for 8 hr at 27°C while progesterone was 43.6 μg/g and 20β-ol was 15.9 μg/g in day 16 tissue. All concentrations differed significantly from endogenous levels ($P < 0.05$) except mean progesterone at day 16 indicating that data obtained from tissue handled in this manner would not accurately represent endogenous concentrations of these progestins.

The relation between stage of pregnancy and progestin concentration of luteal tissue is presented in Table I. The animal with twins is not included in the 170-209 day average. Progesterone and 20β-ol concentrations are in general agreement with recent investigations(2,12,13). However, progesterone concentrations are higher than those reported by

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TABLE I. Bovine Luteal Progesterone and Δ^4 Pregnen-20β-ol-3-one during Pregnancy.

Stage of pregnancy (days)	No. of animals	Mean C.L. wt (g)	Concentration (μg/g) Progesterone Mean	Progesterone Range	20β-ol Mean	20β-ol Range
10– 49	9	4.27	11.6	1.8–29.9	9.2	.0–33.3
50– 89	4	5.80	18.2	1.2–27.4	10.5	2.9–16.9
90–129	9	5.33	6.5	.6–15.0	6.6	.3–17.3
130–169	5	5.62	6.9	1.7–11.2	6.7	4.2– 9.2
170–209	4	7.09	13.2	1.1–29.3	5.3	1.8–12.2
170–209*	1	8.18	33.9		27.8	
210–249	3	7.35	4.0	.6– 6.1	3.3	.3– 5.8
250–280	1	6.16	6.3		4.0	

* Represents an animal with a freemartin twin.

Melampy *et al.*(14) in earlier chemical assays. These differences can probably be attributed to analytical procedures used. In pregnant animals 20β-ol was found to be a greater portion of the 2 progestins than that found in cycling animals. This indicates that 20β-ol may play an important role in maintenance of pregnancy. This substance exhibits 1/5 to 2 $\times$ the activity of progesterone in rabbit and mouse uterine assays(15), but its activity in cows remains to be investigated.

Variability of progesterone content of peripheral blood during pregnancy is shown in Table II. These values, ranging from 0 to 2.13 μg/100 ml plasma, are more variable and have a slightly higher mean than those reported by Short(16). No 20β-ol was detected in the samples analyzed.

Five samples of placental fluids (785-2945 ml/sample) from animals 50-170 days pregnant contained no detectable progesterone or 20β-ol. In 6 placentae (1.54 to 3.06 kg/placenta) from animals 90-280 days pregnant, none contained detectable 20β-ol and only one contained detectable progesterone. This placenta from an animal 90-130 days pregnant contained 11.7 μg progesterone and weighed 2.63 kg.

TABLE II. Progesterone in Bovine Blood Plasma during Pregnancy.

Cow No.	Duration of pregnancy (days)	Concentration (μg/100 ml plasma)
1	50	.68
2	51	.59
3	52	1.24
4	52	1.92
5	92	2.13
6	92	.0
7	97	1.27
8	153	.27
9	156	1.87
10	168	.89
11	185	1.49
12	187	.30
13	204	.68

Results of Uren and Raeside(17) indicate the corpus luteum is necessary to maintain pregnancy in the cow up to about the 200th day of gestation. Progesterone has been detected at the 100th day of pregnancy in peripheral blood from ewes which were ovariectomized on the 60th day of gestation(8). These results suggest an extra-ovarian source of progesterone in this animal which also has a cotyledonary placenta. Human placenta is reported to contain 1 mg progesterone/kg of tissue(6,18) and mare placenta 73 to 250 μg/kg(8). Progestational activity has been observed in extracts of cow placenta in bioassays(8). Using different analytical procedures, Melampy *et al.*(14) have reported cow placenta to contain up to 200 μg progesterone/kg. However, our results and those of Short(8) and Gorski *et al.*(2) suggest bovine placenta is not a source of progesterone or 20β-ol. Progesterone was not detected in a 30 ml sample of plasma obtained from the uterine vein of a pregnant cow(8). Activity observed in bioassays may be due to another substance with progestational activity.

Summary. Mean endogenous progesterone concentrations of bovine luteal tissue increased from 29.2 μg/g at day 8 to 47.2 μg/g at day 16 of the estrous cycle. Δ^4 Pregnen-20β-ol-3-one increased from 0.3 μg/g at day 8 to 2.0 μg/g at day 16. Mean progesterone concentration increased 46.2 μg/g in tissue at

day 8 of the cycle and 53.4 μg/g in tissue from day 16 during 2 hr incubation in Krebs Ringer bicarbonate medium. Increases of 20β-ol concentration on incubation were 2.1 and 12.4 μg/g at days 8 and 16 respectively. Mean concentration of progesterone ranged from 4.0 to 18.2 μg/g and 20β-ol from 3.3 to 10.5 μg/g in luteal tissue at seven stages of pregnancy investigated. Blood plasma from pregnant animals contained 0 to 2.13 μg progesterone/100 ml plasma and no detectable 20β-ol. Neither progesterone nor 20β-ol was detected in placental fluids and only progesterone (4.4 μg/kg) was found in one of 6 placentae.

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Response of Chickens to Prolonged Feeding of Crude "Toxic Fat".* (27100)

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"Toxic fat" is the name applied to certain distilled animal fats whose residue, when added to the diet of chickens, produces hydropericardium and ascites(1). The toxic fraction is located in the non-saponifiable portion of the fat(2-4). Its chemical structure is unknown; however, it has recently been crystallized(5).

Hydropericardium and ascites occur only in chickens, whereas, a reduction in growth is produced in ducks and turkeys(6). Retarded sexual development has also been reported in growing pullets when fed "toxic fat"(7). We have been able to produce hydropericardium, ascites and mortality as previously described in acute toxicity studies when proper concentrations of the crude "toxic fat" were fed to chickens one day of age. Most of the birds died within 5 weeks when fed concentrations of "toxic fat" above 1.0%. When levels of 1.0 or less were fed, the birds survived for longer periods of time, and showed better weight gains. With a longer survival time hydropericardial and ascitic effusions were not as great and testicular hypoplasia became apparent. It seemed desirable, therefore, to establish the response of young male chickens to concentrations of crude "toxic fat" which would permit good growth without causing death.

Methods. Thirty-six, Vantress-White Rock

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