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PROGESTERONE LEVELS IN THE SYSTEMIC PLASMA OF PREGNANT, CYCLING AND OVARIECTOMIZED COWS

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Summary. The levels of progesterone in the systemic plasma of eight normal and two ovariectomized cows were estimated by gas chromatography. During the last month of pregnancy, levels varied mostly in the 2·5 to 7·5 ng/ml range (0·5 to 3·0 ng/ml at calving). For six of the eight cows, there was a significant fall in level during this period; pooling of the data for all eight showed a highly significant fall.

Levels near the time of ovulation, whether or not this was associated with behavioural oestrus, were below 2 ng/ml; they rose and fell during ovulation cycles as corpora lutea grew and regressed. Mean peak level for twenty-two cycles was 9·0 ng/ml and occurred on average 13 days after ovulation. The time of most rapid fall in progesterone level was, on average, 4 days before ovulation.

Mean levels during the first 14 days after oestrus in normal cycles were not different from those during the first 14 days after insemination in early pregnancies; they then declined in the cycles but not during the early pregnancies.

Mean progesterone levels in the plasma of ovariectomized cows up to 200 days after the operation were below 2 ng/ml; levels showed a slow but significant rise during this period.

INTRODUCTION

A study of the concentrations of progesterone in the systemic blood plasma of the normal cow was undertaken as a further contribution to the understanding of the physiological rôle of the hormone in this species (Folley, 1956; Cowie, 1966; Gomes & Erb, 1965). Progesterone levels were also measured in two bilaterally ovariectomized cows to assess the importance of extra-ovarian progesterone.

MATERIALS AND METHODS

Gas-liquid chromatographic (GLC) material

Commercially-prepared column packing materials were used: 3·8% SE 30

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on 80 to 100 mesh Diatoport S (Hewlett-Packard Ltd); 1% QF1 and 1% SE 30, both on 100 to 120 mesh Gas Chrom Q (Pye-Unicam Ltd).

Experimental animals

Blood samples were obtained from eight normal Friesian cows. The collection period began 30 to 40 days before calving (second pregnancy) and ended about 120 days later when seven of the cows had again been pregnant for 30 days. The experimental animals included two pairs of monozygous and one pair of dizygous twins (see Table 1). Each was milked twice daily before calving as part of an unrelated experiment: cows 287, 288, 293, 297 throughout the normal dry period; the others for 1 month before calving. Calves were removed 24 hr after birth. Occasional samples from other normal Friesians were taken during evaluation of the assay.

Two bilaterally-ovariectomized Friesian cows also provided serial blood samples.

Estimation of time of oestrus and ovulation and of cycle length

The time of oestrus was recorded by observation of mounting behaviour by other cows.

The uterus and both ovaries of the eight cows were palpated *per rectum* at 2- to 4-day intervals from about 10 days after calving. Diameters of follicles and corpora lutea were assessed on a graded scale. The day of ovulation was estimated from observations of the disappearance of a follicle and its replacement by a corpus luteum; occasionally the estimate was based on palpation of an ovulation stigma. The occurrence of twin ovulations was recorded. Palpation sometimes indicated the presence of fluid in corpora lutea; this was not regarded as abnormal.

The term 'quiet ovulation' is used to describe ovulation without behavioural oestrus.

Cycle lengths were measured from the time of one ovulation to the next. Lengths of 17 to 24 days were considered normal.

Blood sampling

Blood samples (100 ml) were taken from the jugular veins of each of the eight cows at about 10.00 hours every other day throughout the 7 months (August to February) of the experiment, every tenth sample from each cow being duplicated. Coagulation was prevented by the addition of oxalate and samples were stored in plastic bottles surrounded by ice for up to 1 hr before the plasma was separated by centrifugation at 0° C. Plasma samples were stored at 5° C in the dark for periods up to 2 hr before progesterone extraction.

Plasma samples from the two ovariectomized cows were similarly obtained at various times from 1 to 217 days after operation.

Progesterone assay

Progesterone concentration was determined in 50-ml plasma samples by a method based on those of Short (1958), Heap & Holzbauer (1965) and Schom-

berg, Coudert & Short (1967). It involved extraction of progesterone from the plasma at pH 12 by means of ether, and paper and gas-liquid chromatography (GLC) using 1% QF1 as stationary phase with flame ionization detector.

[7α-^{3}H]Progesterone (12 ng; 0·09 μCi) was added to each plasma sample before extraction and radio-activity of an aliquot of the extract measured immediately before GLC, when an internal standard of 500 ng of 20β-acetoxy-pregn-4-en-3-one was added. Mean recovery of labelled progesterone added to thirty representative plasma samples was 55%. Two GLC columns only were used (for 75 and 65 days, respectively); they were calibrated daily with progesterone standards. An overall calibration equation relating the ratio of the masses of the two steroids to that of the respective peak areas on the recorder charts was calculated for each column and was used to calculate quantities of progesterone in the plasma extracts.

The assay was extensively tested for error.

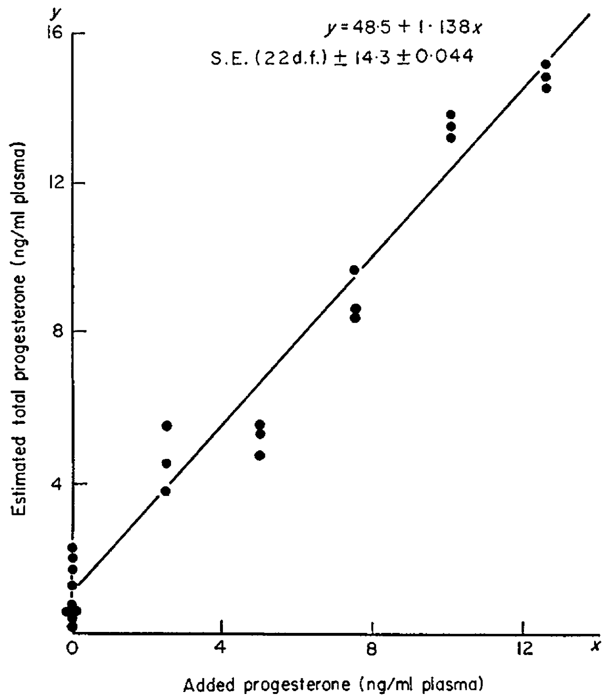
Qualitative evaluation. There is already good evidence for the existence of progesterone in the systemic blood of intact and ovariectomized cows (Gomes & Erb, 1965), but some further specificity tests were carried out.

Treatment of paper chromatograms of extracts of mid-cycle plasma with 2,4-dinitrophenylhydrazine (Rombauts & Piton, 1963) showed products identical in colour and R_F value to those from progesterone and indicated concentrations of about 10 ng progesterone/ml of plasma. Other extracts of pregnancy and non-pregnancy plasma gave GLC peaks of the same R_T as progesterone (0·77 relative to 5α-cholestane on 3% SE 30 and 0·86 relative to 20β-acetoxypregn-4-en-3-one on 1% QF1). An extract from plasma of a cow in the 5th month of pregnancy, treated with ethane-1,2-dithiol (Zmigrod & Lindner, 1966) gave a GLC peak of the same mobility as progesterone bis ethylene thioketal (0·51 on 1% SE 30 relative to the bis ethylene thioketal of androst-4-ene-3,17-dione).

Quantitative evaluation. Progesterone was added to replicate 50 ml plasma samples from the two ovariectomized cows in quantities likely to give total concentrations in the normal range for intact cows. For samples from both cows, there was a linear relationship between added progesterone and estimated total progesterone; percentage bias did not exceed 22% (Text-fig. 1). Mean endogenous progesterone levels obtained directly were 1·0 and 1·7 ng/ml for the two cows, in close agreement with values obtained from the intercepts of the regression lines. Whether the assay had also an element of fixed bias was not determined by these experiments but it may be noted that McCracken (1963) obtained very similar results for ovariectomized cows using a spectrophotometric assay. Tolerance limits of 95% (within which the true value of total progesterone may be expected to lie for a given added quantity of progesterone) were about ±2 ng/ml plasma for both cows.

Further information on precision of the assay was obtained from duplicate analyses for the eight intact cows. Where the error could be pooled (seven cows), a single determination had 95% confidence limits of ±2·2 ng/ml; for the eighth cow the limits were ±0·9 ng/ml.

Calculated error in GLC calibration accounted for about half of these overall errors.



Text-fig. 1. Recovery of progesterone added to 50-ml systemic plasma samples of the ovariectomized cow Wanda: relationship between estimated progesterone y (ng/ml) and added progesterone x (ng/ml).

RESULTS

Plasma progesterone levels for cows in late pregnancy

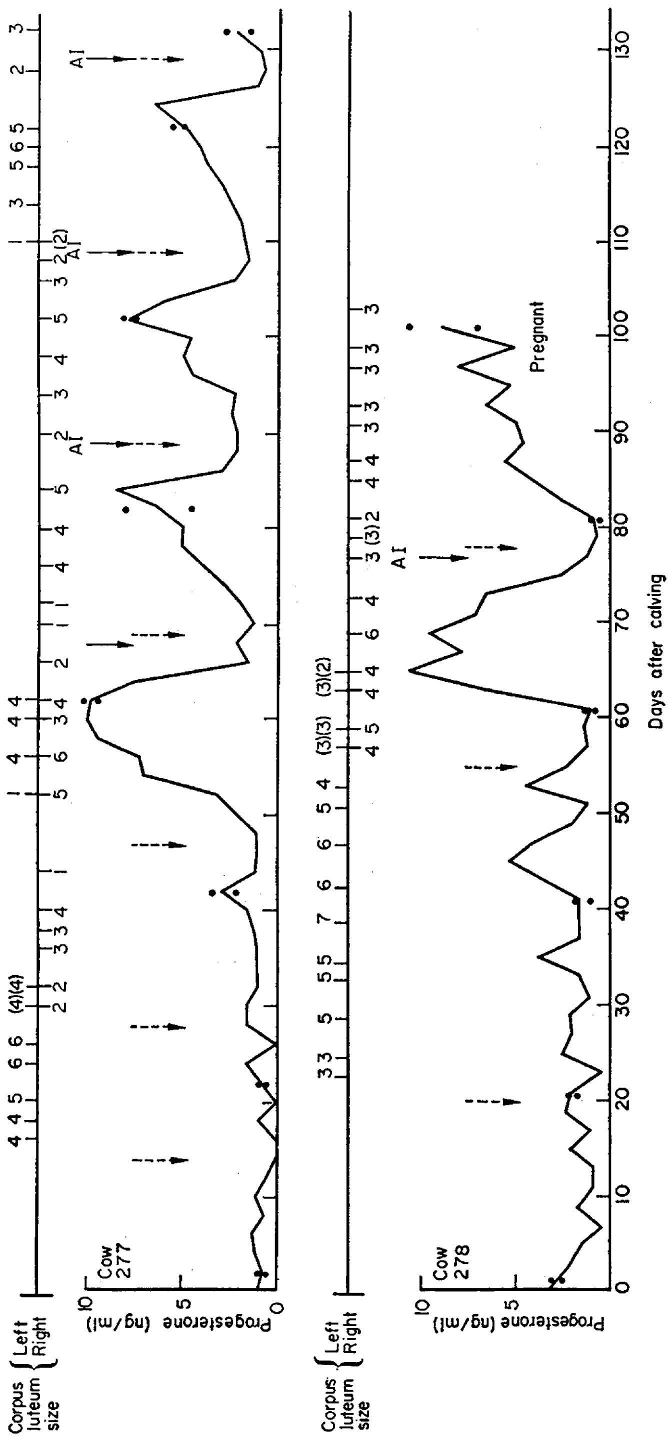
All eight cows showed considerable variation in plasma progesterone levels 30 to 40 days before calving (see Text-fig. 4 for four cows); straight lines, fitted

Table 1

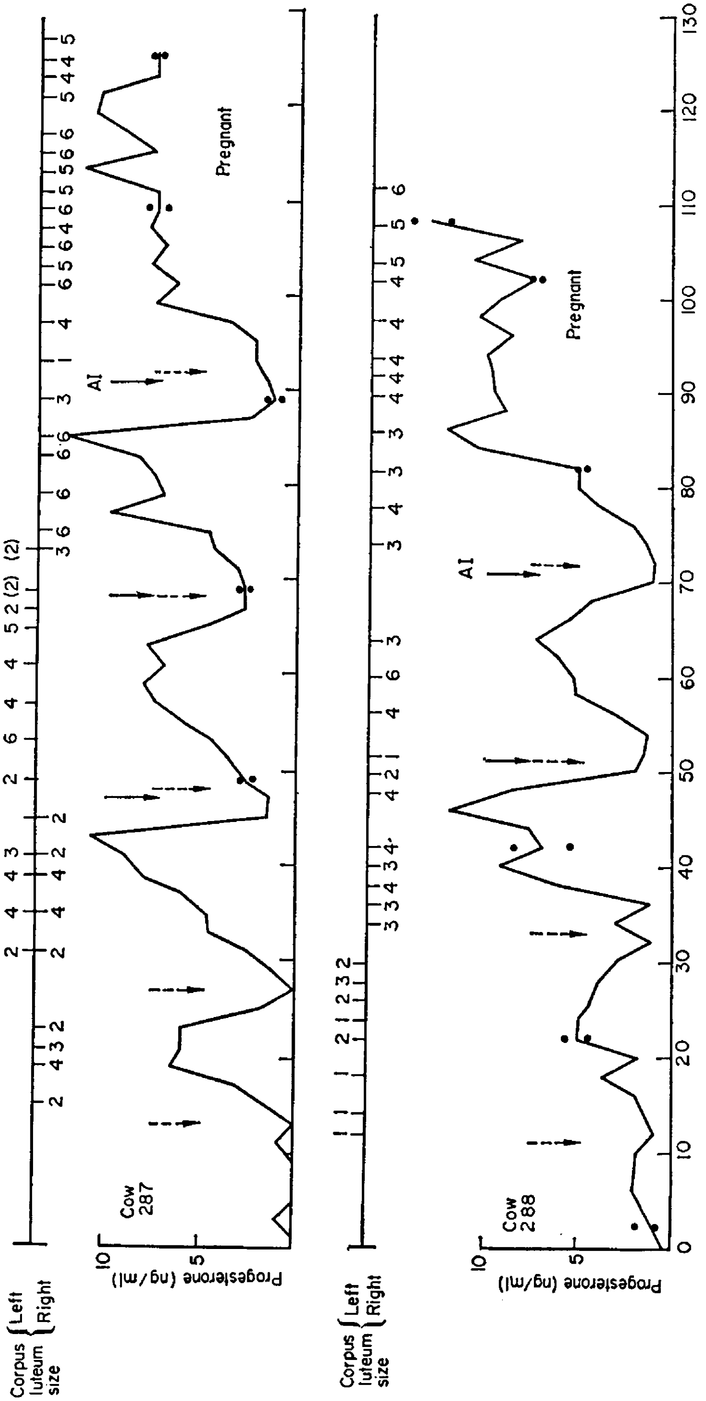
POST PARTUM INTERVAL TO FIRST OVULATION AND FIRST BEHAVIOURAL OESTRUS, AND LENGTHS OF CYCLES IN EIGHT LACTATING COWS

Cow	Post partum *interval (days) to:* *Ovulation*	*Behavioural oestrus*	*Lengths (days) of ovulation cycles*
277 } MZ	14	68	14, 19, 22, (*20, 20, 20*)
278 } MZ	20	77	35, 23
287 } DZ	13	47	14, 21, (*20, 24*)
288 } DZ	11	51	22, 18, (*21*)
293	12	86	12, 23, 17, 22
297 } MZ	20	18	20, 17, (*18*)
298 } MZ	18	34	17, (*22*)
305	22	20	(*22, 23*)

1. Cycles listed in chronological order; italic figures indicate behavioural oestrus at beginning and end of cycle.
2. MZ, monozygous twins; DZ, dizygous twins.
3. For 305, lengths of two cycles not recorded due to break in ovarian palpation routine.



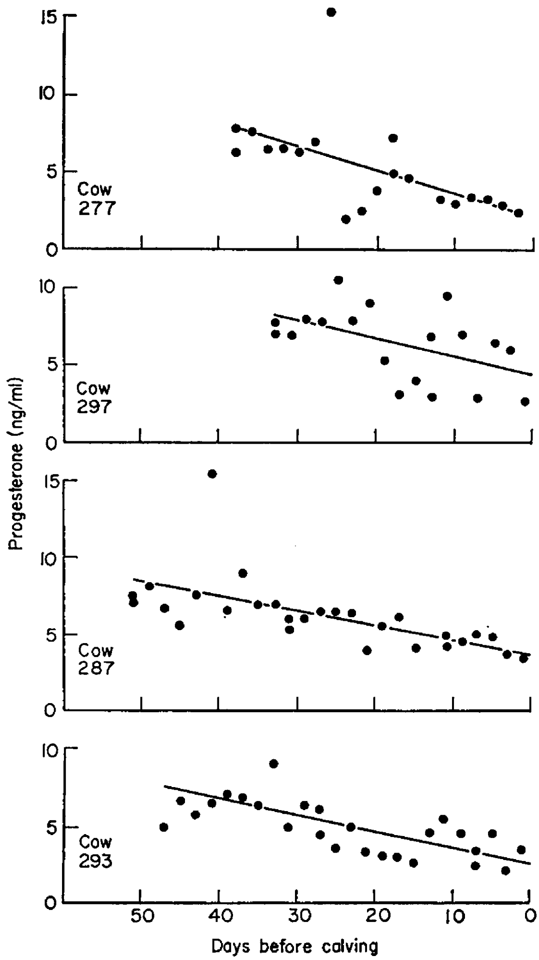
TEXT-FIG. 2. Progesterone levels in the systemic plasma of cows 277 and 278 after calving and during the early pregnancy of cow 278. Solid arrow: behavioural oestrus; broken arrow: ovulation. AI: artificial insemination. Diameter (on graded scale) assessed by palpation of corpus luteum, 1 to 6; corpus albicans, (1) to (6).



TEXT-FIG. 3. Progesterone levels in systemic plasma of cows 287 and 288 after calving and during early pregnancy. Key as Text-fig. 2.

to the estimates in this period for six of the eight cows, had a significantly negative slope ($P<0{\cdot}05$) and the combined regression line for all cows had a highly significant negative slope ($P<0{\cdot}001$). In some cows, the *ante partum* fall seemed to occur more sharply in about the last 5 days of pregnancy.

Between cows, there was only slight variation in the slope of the regression lines but greater variation in intercept, i.e. in absolute levels of plasma progesterone.



TEXT-FIG. 4. Progesterone levels in systemic plasma of cows 277, 297, 287 and 293 before calving.

gesterone. There was no evidence of greater similarity of progesterone levels between monozygous twins than between cows not so related.

Post partum *plasma progesterone levels before normal cycles were re-established*

Calving was normal for all eight cows, except that No. 278 (Table 1 and Text-fig. 2) retained the placenta for 4 days. Ovulation cycles recommenced for all cows by about 20 days *post partum* and were of normal length except for

the first cycles of four cows (Table 1). In one abnormal first cycle (cow 287, Text-fig. 3), a normal corpus luteum developed with normal plasma progesterone levels for 6 to 10 days but it then regressed and a 14-day cycle resulted. In another (cow 278, Text-fig. 2), a large corpus luteum persisted for 35 days but with low plasma progesterone levels.

Plasma progesterone levels at ovulation

It is evident (Text-figs. 2 and 3) that near the time of ovulation plasma levels of progesterone are relatively low. Mean levels at ovulation accompanied by oestrus and at quiet ovulation are not significantly different (Table 2), the overall mean being 1·5 ng/ml.

Quiet ovulation was observed more commonly in the earlier part of the *post partum* period when plasma progesterone levels were often relatively low; its incidence varied greatly between cows (Table 1, Text-figs. 2 and 3). In Table 2, it may be seen that the mean peak progesterone level during the 10 days preceding ovulation with oestrus is much greater than in the same period before quiet ovulation.

TABLE 2

MEAN LEVELS OF PLASMA PROGESTERONE AT NORMAL AND QUIET OVULATION, AND MEAN PEAK LEVELS BEFORE OVULATION IN EIGHT NORMAL COWS

Group	*Nature of ovulation*	*No. of ovulations*	*Mean progesterone level at ovulation (ng/ml)*	*Mean peak progesterone level during 10-day period before ovulation (ng/ml)*
(1)	Normal	20	1·8	9·0
(2)	Quiet	15	1·3	3·7
Difference			0·5[NS]	5·3***
Standard error (with 33 d.f.)			±0·33	±0·96
All ovulations		35	1·5±0·16 (S.E.M.)	

Significance levels: [NS] ($P>0·05$), ** ($P<0·01$), *** ($P<0·001$).

Standard errors are based on variation between ovulations within groups (with 33 d.f.) because non-orthogonal analysis revealed no significant cow differences or interaction between cows and groups.

Progesterone level 'at ovulation' was that for the estimated day of ovulation or the previous day, as data were available.

Plasma progesterone levels in normal cycles

Text-figures 2 and 3 show mid-cycle peaks of 7 to 12 ng progesterone/ml; the level rises and falls during the cycle as the corpus luteum grows and regresses. Ten cycles from the eight cows were of normal length and began and ended with ovulation and oestrus, whereas twelve others had normal length but began or ended, or both, with quiet ovulation. No differences were found in mean plasma progesterone levels for the two groups of cycles (Table 3). From the pooled data, a small but significant increase in the mean level between Days 2 to 3 and 5 to 6 after ovulation was demonstrated.

TABLE 3

MEAN LEVELS OF PLASMA PROGESTERONE DURING OVULATION CYCLES OF NORMAL LENGTH IN EIGHT COWS

Group	*Nature of cycle*	*No. of cycles*	*Cycle length (days) Mean and (Range)*	*Mean progesterone level 2 to 3 days after ovulation (ng/ml)*	*Mean progesterone level 5 to 6 days after ovulation (ng/ml)*	*Mean peak level of progesterone (ng/ml)*	*Mean time of peak progesterone level (as proportion of cycle length)*	*Mean time of greatest fall in progesterone level (days before ovulation)*
(1)	Cycles beginning and ending with oestrus and ovulation	10	21·0 (18 to 24)	2·0	3·2	9·4	0·61	4·6
(2)	Cycles beginning or ending (or both) with quiet ovulation	12	20·1 (17 to 23)	2·3	3·7	8·6	0·66	3·8
Difference			$0{\cdot}9^{NS}$	$-0{\cdot}3^{NS}$	$-0{\cdot}5^{NS}$	$0{\cdot}8^{NS}$	$-0{\cdot}05^{NS}$	$0{\cdot}8^{NS}$
Standard error of difference (with 20 d.f.)			±0·91	±0·49	±0·84	±1·26	±0·049	±0·58
All cycles		22	20·5 ±0·45 (S.E.M.)	2·2	3·5	9·0 ±0·63 (S.E.M.)	0·64 ±0·02 (S.E.M.)	4·2 ±0·29 (S.E.M.)
				Difference: 1·3** S.E. (with 21 d.f.): ±0·38				

Significance levels: ^{NS} ($P>0{\cdot}05$), **($P<0{\cdot}01$).

Plasma progesterone levels in cycles following twin ovulations

On three occasions out of thirty-five, twin ovulations occurred and peak progesterone levels in the ensuing cycles were 10·2, 9·9 and 10·8 ng/ml. The length of the first of these three was not determined owing to quiet ovulation and a break in the ovarian palpation routine, but from plasma progesterone levels it appeared to be normal; the other two were of normal length. Since the mean peak progesterone level for all normal cycles was 9·0 ng/ml ±2·9 (SD), there is no obvious effect of the twin ovulations.

Plasma progesterone levels in early pregnancy

Progesterone levels during the first 14 days of pregnancy did not differ from those during the first 14 days after oestrus in cycles (Table 4). Thereafter, whereas levels in cycles declined, those in pregnancy were maintained.

TABLE 4

MEAN LEVELS OF PLASMA PROGESTERONE (NG/ML) AT COMPARABLE TIMES IN THE OESTROUS CYCLE† AND IN EARLY PREGNANCY IN EIGHT NORMAL COWS

	Early pregnancies	*Cycles*	*Difference* (± *S.E. with* 17 *d.f.*)
Total	7	12	
Mean progesterone levels (ng/ml):			
(a) At oestrus (or previous day)	1·9	2·1	$-0·2^{NS}(\pm 0·45)$
(b) 13 to 14 days after oestrus	$7·4^{(1)}$	7·8	$-0·4^{NS}(\pm 1·09)$
(c) During first 14 days after oestrus	5·0	4·6	$0·4^{NS}(\pm 0·54)$
(d) 20 to 21 days after oestrus	$9·5^{(2)}$	1·5	7·9***(±0·91)
(e) During period 15 to 21 days after oestrus	8·6	4·1	4·5***(±0·87)

Significance levels: ^{NS} ($P > 0·05$), ***($P < 0·001$).
Difference of means (1) and (2): $-2·1^{NS}$.
Standard error of difference (with 12 d.f.): ±1·4.
† Cycle lengths measured from the time of behavioural oestrus.

Plasma progesterone levels in ovariectomized cows

Levels of plasma progesterone were studied in two bilaterally ovariectomized cows, from 45 to 217 days after the operation in one animal and from 1 to 149 days in the other. From Table 5, it is seen that mean levels were below 2 ng/ml for each cow but highly significant rises occurred during the study. There was no evidence of periodic fluctuation in level.

DISCUSSION

Short (1958), using a spectrophotometric assay, reported a fall in systemic plasma levels of progesterone in the cow in the last 14 days of pregnancy. The results of the present study also clearly indicated a fall in levels during the last month. But Erb, Estergreen, Gomes, Plotka & Frost (1968), using a double-isotope derivative assay, found considerably higher levels in the last month of pregnancy in the cow and no fall.

The re-establishment of ovulation cycles soon after calving, with a short first cycle and quiet ovulation more frequent in the early *post partum* period, is

normal for high-yielding dairy cows (Labhsetwar, Tyler & Casida, 1963; Morrow, Roberts, McEntee & Gray, 1966). The exceptionally long first cycle of cow 278 with a persistent corpus luteum might have been associated with some infection of the uterus; this cow had retained the placenta for 4 days and had been treated with antibiotics. Lynn, McNutt & Casida (1966) noted that uterine infection and persistence of the corpus luteum in cattle often occur together clinically, but that this only occurred experimentally when there was bacterial inoculation of the uterus together with absence of suckling and milking. From the relatively low levels of plasma progesterone in this very long first cycle (Text-fig. 2), it appears that the bovine corpus luteum may be maintained at near maximvm size for about 30 days without secreting normal quantities of progesterone. Schomberg *et al.* (1967) noted that prolongation of the life of the corpus luteum is not necessarily associated with full maintenance of progesterone secretion.

Levels of progesterone in the systemic plasma of the cycling, non-pregnant cow reported here are in good agreement with values obtained by McCracken (private communication), who used a spectrophotometric assay, and with those of Schomberg *et al.* (1967) and Shemesh, Ayalon & Lindner (1968), who

TABLE 5

MEAN LEVELS (NG/ML) OF PLASMA PROGESTERONE IN BILATERALLY OVARIECTOMIZED COWS, AND LINEAR REGRESSION COEFFICIENTS RELATING PROGESTERONE LEVEL WITH TIME (DAYS)

Cow	*Period after operation (days)*	*No. of samples assayed*	*Mean progesterone level (ng/ml)*	*Regression coefficient (ng/ml/day)*	*S.E. of coefficient (ng/ml/day)*
Wanda	45 to 217	28	0·65	+0·006***	±0·0016
Regina 20	1 to 149	52	1·28	+0·020***	±0·0032

Significance levels: ***(P<0·001).

used assays similar to the present one. They are substantially lower than those reported by Plotka, Erb, Callahan & Gomes (1967), who used a double isotope derivative assay.

The relatively high plasma levels in mid-cycle are thought to be involved in the inhibition of ovulation; certainly the rapidly-falling level is closely associated with impending ovulation. Hafs & Armstrong (1968) reported very low levels in the corpus luteum on the day after ovulation, but a statistically significant rise both in corpus luteum weight and in its progesterone concentration from the 3rd to the 6th day after ovulation. During this same 3-day period in the present study, there was a significant rise in the plasma progesterone level. Gomes, Estergreen, Frost & Erb (1963) found the highest concentration of progesterone in luteal tissue and the greatest luteal weight 13 days after ovulation, and from the combined data of Gomes *et al.* (1963) and Hafs & Armstrong (1968) it appears that the content of progesterone in the corpus luteum, 17 days after ovulation, has fallen to 30 to 40% of the peak value. The timing of these observations coincides with that of the peak level of plasma progesterone and the time of its greatest fall observed in the present study.

From the results summarized in Table 4, it appears that the conceptus has no influence on plasma progesterone level (nor, by inference, on the secretion of progesterone by the corpus luteum) until the 14th day after insemination; the influence is very marked by the 18th day. Similar results have been reported by Shemesh *et al.* (1968).

The estimates of plasma progesterone following ovariectomy are in agreement with McCracken's results (1963). Levels fell in 24 hr to less than 2 ng/ml and then gradually rose, at times reaching 5 ng/ml. It is reasonable to suppose that the low levels found soon after the operation represent the extra-ovarian contribution in the intact, non-pregnant cow and it may be that the low plasma level of progesterone present at oestrus and ovulation (on average not greater than 2 ng/ml) also arises from extra-ovarian sources.

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