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Selected Factors that Affect the Measurement of Plasma Progesterone Concentrations in Pregnant Ewes

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Abstract

A competitive protein-binding technique was used to measure progesterone concentrations in the peripheral plasma of pregnant ewes. Neither haemolysis of blood nor thawing of plasma samples affected plasma progesterone concentration. Blood samples should be chilled immediately upon collection but subsequent to centrifugation immediate chilling of the plasma samples is not critical. No consistent diurnal variation in progesterone concentrations was evident but there was large apparently random day-to-day variation in progesterone concentrations for any ewe. Although a significant positive correlation was found between endogenous progesterone and corticosteroid concentrations, the present study failed to correlate experimentally elevated plasma corticosteroid concentrations with progesterone concentrations. Progesterone concentrations varied greatly between ewes at the same stage of pregnancy.

Introduction

The development of competitive protein-binding techniques for the measurement of steroids in peripheral plasma (see Murphy 1967; Bassett and Hinks 1969; Johansson 1969) has made a large number of hormonal investigations possible in domestic species, for example in sheep (Bassett *et al.* 1969; Fylling 1970), in cattle (Donaldson *et al.* 1970; Henricks *et al.* 1970) and in pigs (Edqvist and Lamm 1971; Henricks *et al.* 1972).

A number of studies have utilized these analytical techniques to diagnose the reproductive state of farm animals and to investigate instances of reproductive failure. For example, Robertson and Sarda (1971) evaluated the use of changes in progesterone concentration as a basis of an early pregnancy test in ewes, cows and sows. Gadsby *et al.* (1972) extended this technique in an attempt to determine the number of foetuses in pregnant ewes. Progesterone concentrations in the pre-pubertal and lactating gilt have been used by Shearer *et al.* (1972) to evaluate the level of ovarian function. Obst *et al.* (1972) concluded that the study of ovine plasma hormone concentrations may help elucidate the reproductive problems associated with the ingestion of phyto-oestrogens.

The efficacy with which these techniques may be employed depends in part upon an understanding of the physical and biological factors that may influence the measurement of steroids in peripheral plasma. The present study, using a competitive protein-binding technique, is restricted to an investigation of some of the factors that may be important in the precise determination of plasma progesterone concentrations in pregnant ewes and for which there is little data available. The results are part of a

comprehensive study evaluating the use of progesterone concentrations as an indication of the foetal number in pregnant ewes.

Materials and Methods

Animals

Pregnant Merino ewes were either kept at pasture or maintained in individual pens. Stage of pregnancy was calculated either from mating observations (Radford *et al.* 1960) or from the time of lambing; the duration of pregnancy in days ± S.D. for representative single- and twin-bearing ewes was 151·6±2·1 (n = 194) and 151·5±2·0 (n = 157) respectively. All ewes were lambed under surveillance either in individual pens or in a drift system (Giles 1968).

Blood Sampling

All blood samples were collected from a jugular vein. Except where otherwise indicated, samples were collected directly into heparinized centrifuge tubes and immediately placed on crushed ice. Within 3 h of collection the samples were centrifuged and the plasma stored at −10°C until assayed.

The effects on plasma progesterone concentrations of haemolysis purposely produced by forced passage of blood samples through a narrow orifice and of repeated thawing of frozen plasma were investigated.

To evaluate the effect of sample preparation on progestin levels, whole blood, serum and plasma samples were taken from each of three pregnant ewes. The samples were treated as shown in Table 3. Losses at the extraction phase of the assay were determined from the recovery of [^{14}C]progesterone added to samples of whole blood, serum and plasma. In addition, a blood sample was collected from each of 40 ewes at the 4th and 18th weeks of pregnancy. All samples were immediately halved and placed on ice; one sample was centrifuged within 1 h and the duplicate within 4–5 h of collection.

To cover the contingency of failure of freezing facilities, the progesterone concentrations in plasma samples from each of six pregnant ewes following storage of the plasma at 6, 23 or 37°C for 8, 24 or 48 h were compared with the progesterone levels in samples stored continuously at −10°C.

The possible influences of exogenous and endogenous corticosteroids on peripheral plasma progesterone concentration were evaluated. Three ewes, 115–118 days pregnant, were housed in metabolism cages and fed at 0745 h daily. Indwelling sampling (15 cm) and infusion (100 cm) catheters were placed in the jugular veins and blood samples (6 ml) taken twice daily for 4 days. Subsequently, beginning at 0845 h on consecutive days, the ewes were infused for 3-h periods with saline (3·7 ml/h), cortisol succinate (375, 750 or 1500 μg/h), saline (3·7 ml/h) and synthetic adrenocorticotrophic hormone (ACTH) (Ciba) (3, 6 or 12 μg/h). Blood samples were collected at frequent intervals (see Fig. 4) and plasma progesterone and corticosteroid concentrations determined.

Hormone Assays and Evaluation

Progesterone was measured by the competitive protein-binding technique of Bassett and Hinks (1969) as modified by Thorburn and Schneider (1972). The following additional modifications were found to increase assay speed and precision:

1. A single extraction of plasma with 6 ml instead of two extractions each with 4 ml of n-hexane.
2. The incubation period at 40°C was reduced from 30 to 15 min and the pre- and mid-incubation mixings were eliminated.
3. A regression equation of the form $y = a+bx-cx^2$ was used to calculate the progesterone concentrations (x represents the time taken to count 50000 counts in the protein-bound fraction).

Corticosteroids were measured as described by Bassett and Hinks (1969).

The apparent progesterone concentration of wether plasma was 0·17±0·35 ng/ml (n = 15) while that of the hexane blank was 0·08±0·31 ng (n = 5). The large standard deviations observed at these low concentrations of progesterone reflect the fact that the regression equations did not accurately assess the amount of progesterone present at levels less than 0·25 ng/ml of plasma. When tritiated cortisol was added to plasma and subjected to a single extraction with n-hexane (6 ml) followed by washing of the hexane with water (1 ml), 0·50±0·01% (n = 3) was recovered in the hexane; the corresponding value when the plasma was extracted twice with light petroleum (Thorburn *et al.* 1969) was 0·37±0·04% (n = 3).

Assay recovery rates were determined by assaying wether plasma containing known amounts of dissolved progesterone. The results are shown in Table 1. All progesterone values reported in this paper have been corrected to 100% recovery using the mean recovery rates of 84 and 89% obtained for the extraction of 1 and 0·5 ml of plasma respectively (Table 1).

Table 1. Assay recovery of progesterone added to wether plasma

Volume of plasma extracted (ml)	Volume of hexane extract assayed (ml)	Range of progesterone concns (ng/ml)	Recovery rate of progesterone (%)[A]
1·0	1·0	1–8	84±3
0·5	1·0	2–16	89±2
0·5	0·5	16–64	89±6

[A] Each value is the mean (± S.D.) of three or four determinations performed in triplicate at three or four progesterone concentrations within the range stated.

Duplicate determinations of the progesterone concentration of plasma samples were carried out both on a within- and a between-assay basis and the coefficients of variation between duplicates were calculated (Table 2). An analysis of variance using the determined progesterone concentrations of five samples of an internal control plasma in each of 24 assays indicated a significant assay effect ($P < 0·001$).

Table 2. Precision of assay for determination of plasma progesterone in pregnant sheep

Number of duplicate assays is given in parentheses

Mean progesterone concentration (ng/ml plasma)	Coefficient of variation (%) between duplicates: Within an assay	Between assays[A]
3·0	6·0 (33)	10·8 (82)
10·0	6·0 (12)	10·2 (23)
17·0	6·7 (15)	12·2 (70)

[A] Each sample was assayed once in two separate assays and a total of 24 assays were involved.

The index of determination, defined as the regression sum of squares divided by the total sum of squares for each of a series of standard curves, was within the range 0·9826–0·9998. When the regression was calculated on the mean values for two or more standard curves the range reduced to 0·9991–0·9998. The procedure was adopted whereby, for any series of assays, one solution of corticosteroid-binding globulin was prepared, the regression coefficients were calculated from the mean values obtained for the standard curve relationship and the progesterone concentrations so calculated within each assay were multiplied by the mean progesterone concentration of five samples of an internal control plasma and divided by the overall mean progesterone concentration of the same standard assayed throughout the assay series.

To examine the possibility that very high concentrations of corticosteroid may interfere with the measurement of progesterone by the competitive protein-binding technique, 10 plasma samples from ewe 6080 (Fig. 4) that contained corticosteroid concentrations in the range of 15–190 ng/ml were extracted in triplicate with 6 ml of n-hexane, and back-washed with distilled water (1 ml). Redistilled n-hexane (8 ml) was added to a 2-ml aliquot of each extract and the total evaporated and assayed for progesterone. Using 10 ml of ethanol–hexane (1 : 99), a second 2-ml aliquot was

eluted through 0·5 g of alumina (M. Woelm Eschwege) as described by Thorburn and Schneider (1972). At this concentration of ethanol, corticosteroids are not eluted from the alumina column. The 10-ml eluate was evaporated and assayed for progesterone. Where relevant, progesterone concentrations were corrected for a 96% recovery of [1,2-^{3}H]progesterone from the alumina column. No change in the progesterone assay blank was attributable to the alumina chromatographic procedure.

Results

Haemolysis and Thawing of Plasma

There were no significant differences in plasma progesterone concentration between haemolysed and non-haemolysed samples ($t = 1·1$, $n = 28$; one missing value) nor between samples thawed one, three or five times.

Collection of Whole Blood, Serum and Plasma

Recoveries of [^{14}C]progesterone added to whole blood, serum and plasma followed by extraction with hexane and washing with water were $88·2 \pm 1·5$, $91·2 \pm 0·4$ and $90·4 \pm 1·3$% respectively ($n = 3$) in the hexane.

Table 3. Progestin concentration in plasma, serum and whole blood samples

Values are means of triplicate determinations. For each of groups 1, 2 and 3 the within-ewe values having different postscripts (a, b, c or d) are significantly different ($P < 0·05$). For group 4 the comparisons are as stated in the table

Sample treatment	Mean progestin concentration (ng/ml): Ewe 6053	Ewe 6119	Ewe 6082
1. Whole blood			
A. Heparinized, placed in ice and frozen after 15 min	11·6 a	10·9 a	11·1 a
B. Left at room temperature (RT), 20–25°C, for 4 h then frozen	7·5 b	8·7 b	9·4 b
C. Left at RT for 20 h then frozen	4·0 c	6·1 c	6·8 c
2. Serum			
D. Allowed to separate at RT for 2 h then frozen	9·4 a	9·9 a	11·3 a
E. Allowed to separate at RT for 4 h then frozen	10·7 a	8·7 b	12·9 b
F. Allowed to separate at RT for 20 h then frozen	9·9 a	5·9 c	6·7 c
3. Plasma			
G. Blood sample immediately placed in ice and centrifuged (5°C) within 15 min. Plasma frozen immediately following separation	11·1 a	10·6 a	9·2 a
H. Blood sample left at RT for 4 h prior to centrifugation as for G	8·6 a	8·5 b	10·4 b
I. Blood sample left at RT for 20 h prior to centrifugation as for G	3·3 b	4·7 c	7·0 c
4. Plasma			
J. Blood sample left at RT prior to centrifugation as for G. Compared with G treatment	9·3 a	9·3 d	10·1 a
K. As for G but centrifuged at RT. Compared with G treatment	10·3 a	12·0 a	9·8 a
L. As for J but separated plasma not frozen until 4 h after separation. Compared with J treatment	8·8 a	11·6 d	10·5 a

Different methods of handling the blood samples after collection were investigated by analysis of variance. The analysis was on a within-ewe basis, separate analyses being performed for treatment groups 1, 2 and 3 (see Table 3). For whole blood, sera and plasma, except in the case of the serum samples from ewe 6053, the effect of treatment on progestin concentration was highly significant ($P < 0{\cdot}001$).

A test of the difference between mean progestin concentrations with Tukey's test showed that, for each ewe, the progestin concentration of whole blood left at room temperature (RT) for either 4 or 20 h was lower than that of whole blood frozen after chilling for 15 min ($P < 0{\cdot}05$). The effect on progestin concentration of subjecting the blood sample to RT for 4 h prior to separation of sera compared with the effect of the same treatment for 2 h was variable but, at least for two of the three ewes, similar treatment for 20 h resulted in significantly decreased progestin concentrations ($P < 0{\cdot}05$). The progestin concentration of plasma obtained subsequent to blood being left at RT for 4 h compared with the progestin concentration of plasma obtained from blood that had been chilled for 15 min was significantly decreased in two of three instances ($P < 0{\cdot}05$) and was significantly decreased in three of three instances if the blood was allowed to stand at RT for 20 h ($P < 0{\cdot}05$).

Analysis of variance of the effects of processing the blood samples in group 4 indicated that, in one of three instances, leaving the blood sample at RT for 15 min prior to centrifuging significantly decreased the progestin concentration of the separated plasma ($P < 0{\cdot}05$). In no instances was there any effect of centrifuging at RT versus centrifuging at 5°C or of leaving the separated plasma at RT for 4 h prior to freezing versus immediate freezing following centrifugation.

At the 4th and 18th weeks of pregnancy 40 ewes were bled and plasma samples were obtained from blood chilled and centrifuged within 1 h of collection or from blood similarly chilled but not centrifuged until 4–5 h after collection. The plasma progesterone concentrations were not significantly different at either stage of pregnancy ($2{\cdot}8 \pm 0{\cdot}8$, $2{\cdot}9 \pm 0{\cdot}6$ and $15{\cdot}6 \pm 6{\cdot}3$, $15{\cdot}8 \pm 6{\cdot}2$ ng/ml respectively).

Storage of Plasma

Storage of plasma at either 6°C for 24 h or at 37°C for 48 h resulted in significantly lowered progesterone concentrations ($P < 0{\cdot}05$) compared with the progesterone concentration of plasma stored continuously at −10°C before assay (Table 4).

Table 4. Effect of storage conditions on plasma progesterone concentrations

Aliquots of plasma samples taken from six pregnant ewes were subjected to various temperature × time treatments prior to refreezing at −10°C. The progesterone concentration of each aliquot was compared to that of plasma stored continuously at −10°C before assay

Storage temperature (°C)	−10	6	6	6	23	23	23	37	37	37
Storage time (h)		8	24	48	8	24	48	8	24	48
Mean progesterone concn (ng/ml) ($n = 6$)	6·8	6·9	6·6	6·8	6·6	6·3	6·7	6·5	6·5	6·0
t value ($t_{0.5} = 2{\cdot}57$)		0·19	2·59	0·21	0·72	2·38	0·98	0·84	0·85	3·12

Diurnal Variation in Peripheral Plasma Progesterone Concentration in Pregnant Merino Ewes

Variation over a 12-h period in peripheral plasma progesterone concentration is shown in Fig. 1. At 47 and 125 days of pregnancy there was no significant difference

in progesterone concentration between each of the four sampling times whereas, at 112 days of pregnancy, the concentration of progesterone at 1200 h was significantly ($P < 0{\cdot}05$) greater than that at 0800 h. At each stage of pregnancy the between-animal variation in progesterone concentration was significant ($P < 0{\cdot}01$).

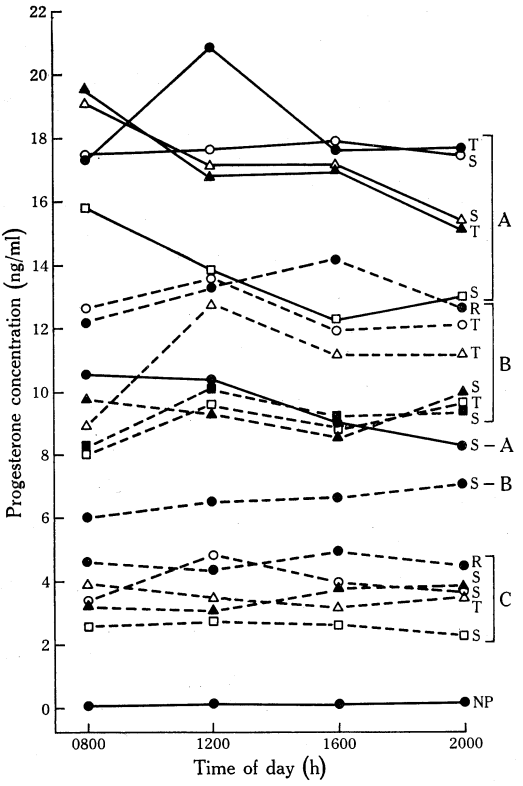

Fig. 1. Variation in the progesterone concentration in the peripheral plasma of pregnant Merino ewes. Stage of pregnancy for ewes in groups A, B and C was 125 ± 1, 112 ± 2 and 47 ± 0 days respectively. Postscripts indicate the number of lambs born to each ewe: *S*, single; *T*, twins; *R*, triplets. *NP*, ewe not pregnant.

Daily Variation in Peripheral Plasma Progesterone Concentration in Pregnant Merino Ewes

Figs 2 and 3 illustrate the day-to-day variation in peripheral plasma progesterone concentration in pregnant Merino ewes bled at 1000 h on each of 10 days with the exception of a 2-day lapse between the fifth and sixth bleeding represented in Fig. 3. Plasma corticosteroid concentrations are also presented in Fig. 3.

At each of the periods beginning at 50, 75 and 116 days of pregnancy (Fig. 2) there were no significant differences in plasma progesterone concentration between each of the 10 days of sampling. These animals were individually penned at Prospect Laboratories (eastern New South Wales). In contrast, in those ewes bled at Trangie Research Station (central western New South Wales) over the period between 114 and 126 days of pregnancy there was a significant ($P < 0{\cdot}01$) effect of day of sampling upon progesterone concentrations. Progesterone concentrations at day 8 were significantly greater ($P < 0{\cdot}01$) than at all other days except for days 5 and 3; those at day 5 were significantly greater than those at day 2. Except in the case of one of the eight ewes bled between days 114 and 126 of pregnancy, there was a positive linear correlation between the plasma progesterone and corticosteroid concentrations

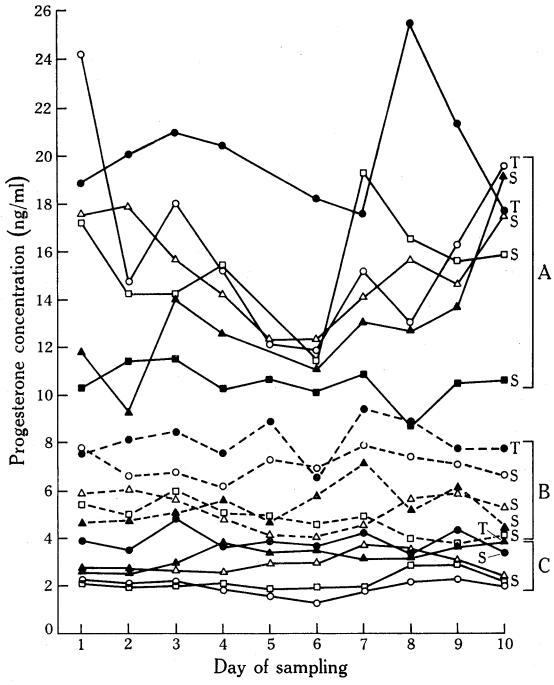

Fig. 2. Daily variation in the progesterone concentration in peripheral plasma in three groups of pregnant Merino ewes in eastern New South Wales. Stage of pregnancy for ewes at the commencement of the sampling period in groups A, B and C was 116 ± 1, 75 ± 0 and 50 ± 2 days respectively. Postscripts indicate the number of lambs born to each ewe: *S*, single; *T*, twins.

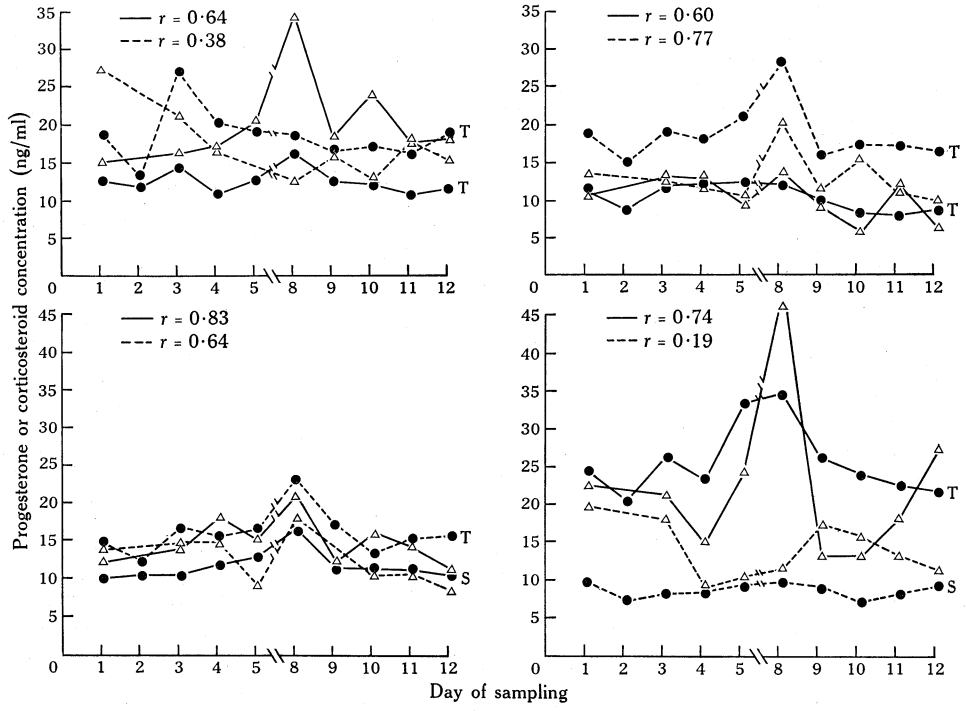

Fig. 3. Daily variation in progesterone (●) and corticosteroid (△) concentrations in peripheral plasma of pregnant Merino ewes in central western New South Wales. Ewes were 114 days pregnant at the commencement of sampling. Linear correlation coefficients (*r*) between progesterone and corticosteroid concentrations for each ewe are also presented. Postscripts indicate the number of lambs born to each ewe: *S*, single; *T*, twins.

(see Fig. 3), the total pooled correlation coefficient ($r = 0{\cdot}63$) being highly significant ($P < 0{\cdot}001$).

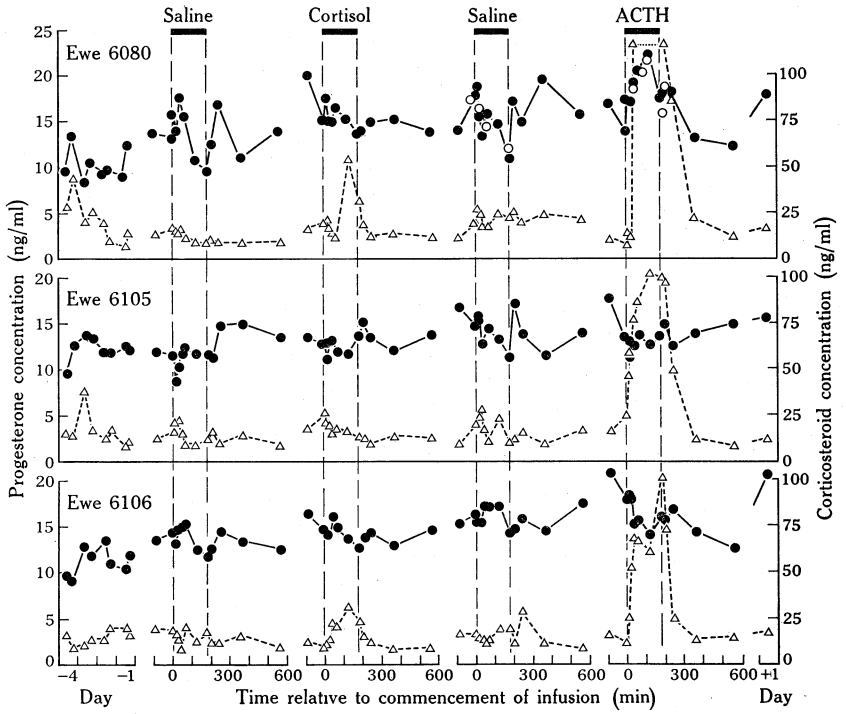

Fig. 4. Changes in the concentrations of progesterone (●) and corticosteroids (△) prior to and during 3-h intravenous infusions of saline, cortisol succinate and synthetic ACTH via a jugular vein. Saline was infused at the rate of 3·5 ml/h. Rates of infusion of cortisol succinate and ACTH were respectively 375 and 12 μg/h for ewe 6080, 1500 and 6 μg/h for ewe 6105 and 750 and 3 μg/h for ewe 6106. Progesterone concentrations subsequent to alumina chromatography (ewe 6080, ACTH infusion period) are also presented (○).

Effect of Infusions of Saline, Cortisol and ACTH on the Circulating Progesterone Level in Pregnant Ewes

Intravenous infusion of saline at 3·7 ml/h over a 3-h period did not affect the concentrations of either corticosteroids or progesterone in pregnant ewes (Fig. 4). However, as was evident in the plasma samples collected twice daily prior to commencement of infusion, the circulating progesterone level in each ewe varied considerably between samplings. No change in plasma progesterone concentration was evident during the infusion of cortisol succinate at 375, 750 or 1500 μg/h. Extremely high concentrations of corticosteroids were present during the infusion of synthetic ACTH. With the possible exception of a very transient peak in progesterone concentration 120 min after the commencement of ACTH infusion at 12 μg/h, there was no systematic change in plasma progesterone concentrations attributable to the treatment. In only 1 of 10 plasma samples was there a significant difference ($P < 0{\cdot}05$) between the routinely determined progesterone concentration and that obtained following the alumina chromatographic step to remove corticosteroids (15·8 and

14·3 ng progesterone/ml respectively). The corticosteroid concentration in this plasma sample was 17 ng/ml, which was within the normal range of circulating corticosteroid concentrations.

Discussion

The modifications reported in this study to the progesterone assay technique of Thorburn and Schneider (1972) were found to increase assay speed without loss of accuracy and precision. Previous findings (Thorburn *et al.* 1969; Thorburn and Mattner 1971) that plasma corticosteroids did not interfere with the progesterone assay were found to apply to the modified technique and were extended to plasma samples in which corticosteroid concentrations were 10 times greater than those normally present in the plasma of pregnant ewes.

Neither haemolysis of blood samples nor repeated thawing of plasma samples had a significant effect on determined plasma progesterone concentrations. Storage of blood at room temperature prior to centrifugation resulted in decreased progesterone concentrations. Therefore, whole blood should be maintained in a chilled state prior to centrifugation. These observations confirm those of Robertson and Sarda (1971) and Johansson (1969) who found that plasma progesterone concentrations in the blood of the cow, ewe, sow and human were not affected by leaving the uncentrifuged blood sample at 4°C for up to 72 h or at 6°C for 48 h. The disappearance of progesterone from unchilled whole blood might be due to conversion of progesterone to metabolites such as 20α-dihydroprogesterone by a 20α-hydroxysteroid dehydrogenase found by Van der Molen and Groen (1968) to be present in erythrocytes. Thorburn *et al.* (1969) have shown that 20α-dihydroprogesterone competes only weakly in this assay system. In situations distant from the laboratory, the collection of serum is often more convenient than the collection of plasma as the latter requires centrifugation facilities. Because of the degradation of progesterone in whole blood, the use of serum samples for determination of progesterone concentration may be of limited value as adequate separation of serum from blood cells proceeds slowly and is incomplete while the blood is chilled.

The results obtained from pregnant Merino sheep indicated that, in general, there is no consistent pattern of change in peripheral progesterone concentrations during the day and that any diurnal variation encountered is far less than the large differences found in progesterone concentration at any one time between individual ewes. A similar result was obtained from ewes at four different phases of the oestrous cycle (Thorburn *et al.* 1969). There is one differing report of diurnal variation in the concentration of progesterone in the peripheral plasma of pregnant women. Johansson (1969) reported greater progesterone concentrations at 0800 h while the patients were still in bed than at any other time of the day. On the other hand, no significant diurnal variation in progesterone concentration was found in either ambulatory patients (Johansson 1969) or in hospitalized patients (Greig *et al.* 1962; Wiest 1967; Fylling 1970).

While there was no consistent pattern of variation in the progesterone concentration attributable to the day of sampling within each of the three 10-day periods of pregnancy for the groups of ewes located at Prospect, large inconsistent fluctuations were a feature of the results. This variation was particularly high in those ewes sampled between 116 and 126 days of pregnancy. These findings agree with the

earlier results of Bassett *et al.* (1969) in sheep and those of Greig *et al.* (1962) in humans. As was evident in the study of diurnal variation, large between-animal differences in progesterone concentration were found in each of the four groups ($P < 0{\cdot}01$).

The positive correlation between progesterone and corticosteroid concentrations within individual ewes suggests a possible interaction between the adrenal function and the plasma progesterone concentration of pregnant ewes. Nevertheless, continuous 3-h infusions of either cortisol succinate or synthetic ACTH into the jugular vein of pregnant ewes failed to elicit any systematic change in the plasma progesterone concentrations. Fylling (1971) found no increase in progesterone concentration when corticosteroid concentrations in the range of 40–80 ng/ml were attained in pregnant ewes by a single intravenous injection of synthetic ACTH. Thus it seems unlikely that the stress associated with the sampling procedure contributed to the large day-to-day variation in progesterone concentrations in either ewes unaccustomed to the sampling procedure or ewes frequently sampled via an indwelling jugular catheter.

The large variation between consecutive daily samplings in the progesterone concentrations in peripheral plasma can probably, at least to some extent, be explained by the short half-life of progesterone in blood and by variation in ovarian and uterine blood flow. The rapid disappearance of progesterone from ovine plasma is well established (Short and Eton 1959; Short and Rowell 1962; Stupnicki *et al.* 1969). Thorburn and Mattner (1971) found that the concentration of progesterone in the peripheral plasma during the luteal phase of the oestrous cycle reflected the differences in secretion rate of progesterone. However, it may not be valid to extrapolate this finding to the pregnant state as Challis *et al.* (1971) found that, in a single ewe, while concentrations of progesterone in uterine venous blood decreased during the last week of pregnancy, the jugular levels did not fall until 48 h before parturition and in fact, at term, the concentrations in uterine and jugular blood were similar. Meschia *et al.* (1967) found that the flow rate through the uteroplacental circuit varied from sheep to sheep over a very wide range (138–492 ml per kilogram per minute) without any systematic relationship to stage of pregnancy over the range 102–139 days.

The short half-life of progesterone coupled with a large variation in uteroplacental blood flow may result in considerable 'hunting' about the steady state by the homeostatic mechanisms postulated by Short and Moore (1959) to control progesterone concentrations in pregnant animals.

Acknowledgments

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References

Bassett, J. M., and Hinks, N. (1969). Micro-determination of corticosteroids in ovine peripheral plasma: effects of venipuncture, corticotropin, insulin and glucose. *J. Endocrinol.* **44**, 387–403.

Bassett, J. M., Oxborrow, T. J., Smith, I. D., and Thorburn, G. D. (1969). The concentration of progesterone in the peripheral plasma of the pregnant ewe. *J. Endocrinol.* **45**, 449–57.

Challis, J. R. G., Harrison, F. A., and Heap, R. B. (1971). Uterine production of oestrogens and progesterone at parturition in the sheep. *J. Reprod. Fertil.* **25**, 306–7.

Donaldson, L. E., Bassett, J. M., and Thorburn, G. D. (1970). Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation, and the effects of undernutrition on exogenous progesterone concentration. *J. Endocrinol.* **48**, 599–614.

Edqvist, L. E., and Lamm, A. M. (1971). Progesterone levels in plasma during the oestrous cycle of the sow measured by a rapid competitive protein-binding technique. *J. Reprod. Fertil.* **25**, 447–9.

Fylling, P. (1970). Disappearance rate of progesterone following simultaneous removal of the corpus luteum and the foeto-placental unit in women. *Acta Endocrinol.* **65**, 284–92.

Fylling, P. (1971). Premature parturition following dexamethasone administration to pregnant ewes. *Acta Endocrinol.* **66**, 289–95.

Gadsby, J. E., Heap, R. B., Powell, D. G., and Walters, D. E. (1972). Diagnosis of pregnancy and of the number of foetuses in sheep from plasma progesterone concentrations. *Vet. Rec.* **90**, 339–43.

Giles, J. R. (1968). A comparison of two lambing management systems. *Proc. Aust. Soc. Anim. Prod.* **7**, 235–8.

Greig, M., Coyle, M. G., Cooper, W., and Walker, J. (1962). Plasma progesterone in mother and foetus in the second half of human pregnancy. *J. Obstet. Gynaecol. Br. Commonw.* **69**, 772–6.

Henricks, D. M., Dickey, J. F., and Niswender, G. D. (1970). Serum luteinizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. *Biol. Reprod.* **2**, 346–51.

Henricks, D. M., Guthrie, H. D., and Handlin, D. L. (1972). Plasma estrogen, progesterone and luteinising hormone levels during the estrous cycle in pigs. *Biol. Reprod.* **6**, 210–18.

Johansson, E. D. B. (1969). Progesterone levels in peripheral plasma during the luteal phase of the normal human menstrual cycle measured by a rapid competitive protein-binding technique. *Acta Endocrinol.* **61**, 592–606.

Meschia, G., Cotter, J. R., Marowski, R. L., and Barron, D. H. (1967). Simultaneous measurement of uterine and umbilical blood flows and oxygen uptakes. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **52**, 1–18.

Murphy, B. E. P. (1967). Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein-binding radioassay. *J. Clin. Endocrinol. Metab.* **27**, 973–90.

Obst, J. M., Seamark, R. F., and McGowan, C. J. (1972). Plasma hormone levels during pregnancy in ewes grazing oestrogenic clover. *Proc. Aust. Soc. Anim. Prod.* **9**, 204–8.

Radford, H. M., Watson, R. H., and Wood, G. F. (1960). A crayon and associated harness for the detection of mating under field conditions. *Aust. Vet. J.* **36**, 57–66.

Robertson, H. A., and Sarda, I. R. (1971). A very early pregnancy test for mammals: its application to the cow, ewe and sow. *J. Endocrinol.* **49**, 407–19.

Shearer, I. J., Purvis, K., Jenkin, G., and Haynes, N. B. (1972). Peripheral plasma progesterone and oestradiol-17β levels before and after puberty in gilts. *J. Reprod. Fertil.* **30**, 347–60.

Short, R. V., and Eton, B. (1959). Progesterone in blood. III. Progesterone in the peripheral blood of pregnant women. *J. Endocrinol.* **18**, 418–25.

Short, R. V., and Moore, N. W. (1959). Progesterone in blood. V. Progesterone and 20α-hydroxy-pregn-4-en-3-one in the placenta and blood of ewes. *J. Endocrinol.* **19**, 288–93.

Short, R. V., and Rowell, J. G. (1962). The half-life of progesterone in the peripheral blood of a ewe at two stages of gestation. *J. Endocrinol.* **25**, 369–74.

Stupnicki, R., McCracken, J. A., and Williams, K. I. H. (1969). Progesterone metabolism in the ewe. *J. Endocrinol.* **45**, 67–74.

Thorburn, G. D., and Mattner, P. E. (1971). Anastomosis of the utero-ovarian and anterior mammary veins for collection of utero-ovarian venous blood: progesterone secretion rates in cyclic ewes. *J. Endocrinol.* **50**, 307–20.

Thorburn, G. D., and Schneider, W. (1972). The progesterone concentration in the plasma of the goat during the oestrous cycle and pregnancy. *J. Endocrinol.* **52**, 23–36.

Thorburn, G. D., Bassett, J. M., and Smith, I. D. (1969). Progesterone concentration in the peripheral plasma of sheep during the oestrous cycle. *J. Endocrinol.* **45**, 459–69.

Van der Molen, H. J., and Groen, D. (1968). Interconversion of progesterone and 20α-dihydro-progesterone and of androstenedione and testosterone in vitro by blood and erythrocytes. *Acta Endocrinol.* **58**, 419–44.

Wiest, W. G. (1967). Estimation of progesterone in biological tissues and fluids from pregnant women by double isotope derivative assay. *Steroids* **10**, 279–90.

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