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PERIPHERAL PLASMA PROGESTERONE AND OESTRADIOL-17β LEVELS BEFORE AND AFTER PUBERTY IN GILTS

I. J. SHEARER, K. PURVIS, G. JENKIN AND N. B. HAYNES

Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire

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Summary. A double isotope derivative assay and a simple competitive protein-binding assay were used to measure peripheral plasma progesterone levels during the pig oestrous cycle. In view of the similarity between the results, the more convenient protein-binding method was employed to determine progesterone levels during prepuberty, the oestrous cycle, early and late pregnancy, parturition and lactation. Peripheral plasma oestradiol-17β levels were measured during the oestrous cycle, early pregnancy and around the time of parturition.

INTRODUCTION

Recent studies have indicated that the simplified competitive protein-binding assay (CPB) as described by Johansson (1969) for the determination of progesterone in the peripheral blood of women may be adapted for the sheep (Bassett, Oxborrow, Smith & Thorburn, 1969) and the cow (Edqvist, Ekman, Gustafsson & Åström, 1970). Its use has also been reported for the assay of progesterone in pig peripheral plasma during the oestrous cycle (Edqvist & Lamm, 1971).

The pig presents problems with regard to the collection of peripheral plasma at frequent intervals over long periods of time. This communication outlines a convenient method of catheterization which allows blood to be taken from animals daily for periods of up to 8 months. Plasma was collected by this method during a longitudinal study from prepuberty to lactation, and progesterone levels were measured by a rapid protein-binding assay after a comparison of the method with a more complicated double isotope derivative (DID) procedure. Peripheral plasma oestradiol-17β levels were also determined at some stages of the reproductive process by a modification of the assays described by Korenman, Perrin & McCallum (1969) and Corker & Exley (1970).

MATERIALS AND METHODS

Animals

Nine crossbred gilts (Landrace × Large White) were used. Prepubertal animals were examined and selection was based on those which were con-

sidered to have a minimum of two ear veins suitable for catheterization. Animals were fed 1·82 kg/day as a standard ration currently used in the pig-breeding unit except during lactation when the amount was increased to 5 kg/day. They were given free access to water for three periods of 15 min throughout the day. Oestrus was determined by visual appraisal and the reaction of the animals to hand pressure on the haunches. Matings were with a stock boar on the late afternoon of the 1st day of oestrus (Day 0) and the following morning. During collection periods, animals were housed in metabolism crates as described by Heap (1966) and were kept under a regimen of 12 hr light/12 hr dark. When not on collection, the animals were housed in individual sow stalls.

Catheterization and blood collection

The jugular vein was catheterized in prepubertal animals (80 to 100 kg body weight) by way of a suitable ear vein, using a modification of the procedure described by Anderson & Elsley (1969). A proprietary catheter (Portex, internal diameter 1·00 mm, length 40 cm; Portex Ltd, Kent) was introduced into the vein as follows: the head of the animal was immobilized by placing a rope tightly round the upper jaw and securing it. A subcutaneous injection of 1 ml 2% xylocaine (Astra-Hewlett Ltd) was given at the proposed site of catheter insertion. A syringe needle was introduced into the ear vein and a flexible wire (Seldinger, 1953) twice the length of the catheter was introduced into the vein through the needle to the depth intended for the catheter and the needle was then removed. The catheter was fed over the protruding half of the wire and eased down the vein until fully inserted. The wire was then withdrawn and the patency of the catheter checked by withdrawing blood with a syringe. If satisfactory, the catheter was rinsed with heparinized saline and a nylon stylette fitted with a rubber bung, small enough to fit the catheter hub, was introduced into the catheter. The ear was then firmly taped and the catheter strapped down on to the existing ear tape. Catheters remained patent for varying lengths of time from 2 weeks to 8 months. During collections, up to 40 ml blood were withdrawn each day while the animals were fed. The blood was collected into 5% of its volume of an aqueous solution containing ammonium oxalate (2·4 g/100 ml) and potassium oxalate (1·6 g/100 ml), centrifuged immediately and the plasma stored at −15° C for subsequent analysis.

Double isotope derivative assay for progesterone

The isotopes—[4-^{14}C]progesterone (36·1 mCi/mM), [7-^{3}H]progesterone (9·2 Ci/mM) and tritiated water (1·0 Ci; 10 Ci/mM) absorbed on basic alumina (3 g)—were obtained from the Radiochemical Centre, Amersham, and used without further purification. All solvents were Analar grade.

The method involved a double isotope procedure utilizing alumina containing tritiated water as the labelling agent. It was carried out as described by Siiteri, Tippit, Yates & Porter (1968) for rat ovarian vein plasma with the following modifications to increase sensitivity. The [4-^{14}C]progesterone was not added to the initial plasma samples before extraction and tritiation since added [4-^{14}C] progesterone is tritiated together with non-radioactive progesterone and its low specific activity with regard to ^{14}C results in high blank values. To eliminate

this, high specific activity [7-^{3}H]progesterone (about 1000 counts/min in 0·1 ml ethanol was added to plasma samples (5 ml plasma diluted with 10 ml distilled water) and a sample removed after extraction and before tritiation to give an estimate of recoveries. The [4-^{14}C]progesterone (about 6000 counts/min in 0·1 ml ethanol) was added immediately before removal of the alumina by filtration. After three purifications by thin layer chromatography (systems I, benzene: ethyl acetate 3:2 v/v; II, benzene:ethyl acetate 4:1 v/v; III, benzene:chloroform 1:1 v/v) the samples were crystallized to constant ^{3}H/^{14}C ratio from acetone/hexane (usually four crystallizations). Plasma progesterone concentrations were obtained by comparing the final ^{3}H/^{14}C ratio of samples after correction for [7-^{3}H]progesterone added to determine recoveries, with a standard curve prepared from a range of progesterone standards subjected to the same procedures.

Protein-binding assay of progesterone

The technique was based on the rapid method described for human plasma by Johannson (1969) and involved the following basic procedures: (a) extraction of 1·0-ml samples of plasma with two 3-ml quantities of petroleum ether (Mallencrodt, boiling range 30 to 60° C); (b) concentration of the extract; (c) binding reaction using 1·0 ml of an aqueous solution of 2·5% male beagle plasma. ([1,2-^{3}H]Corticosterone (40 Ci/mM), obtained from the Radiochemical Centre, Amersham, was used as tracer. One ml of binding protein solution contained about 20,000 counts [1,2-^{3}H]corticosterone/min); (d) separation of bound and unbound fractions by absorption of the unbound steroid on to florisil; (e) radioactive counting of the bound fraction. A mean recovery value was established by adding [7-^{3}H]progesterone to thirty-six samples of pig plasma before the extraction. A standard curve over the range 0 to 12 ng was prepared with each daily assay.

Oestradiol-17β determination

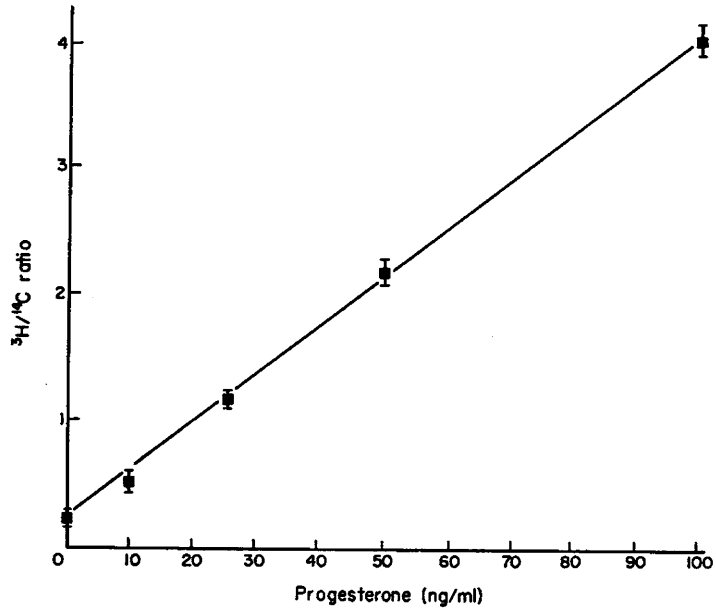
This was carried out using a combination of the methods described by Korenman *et al.* (1969) and Corker & Exley (1970). All glassware was silanized before use by placing it in a mixture of 5·0% trimethyl silyl ether in benzene for 15 min after which it was washed in methanol and distilled water. Plasma samples (1·0 ml) were extracted with two 3-ml quantities of ethyl acetate.

Purification of plasma extracts was carried out by thin layer chromatography with benzene:methanol (20:1) as the developing solvent and using silica-coated plates previously washed by developing in benzene:dichloromethane (1:1). Oestradiol-17β was located by running radioactive markers in adjacent lanes on the thin layer plates and determining the position of this with a Tracerlab thin layer scanner Model no. SC-525.

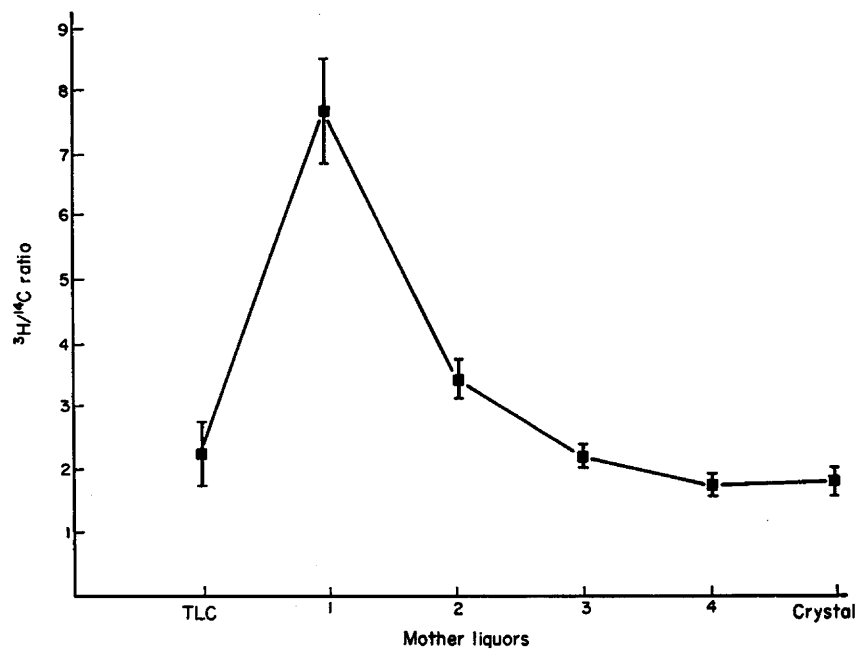
RESULTS

Assay data

A typical standard curve for the DID assay and the results of successive crystallizations on the ^{3}H/^{14}C ratio for a plasma sample assayed in duplicate are



TEXT-FIG. 1. Standard curve for a double isotope derivative assay. Vertical bars indicate the range of duplicate determinations.



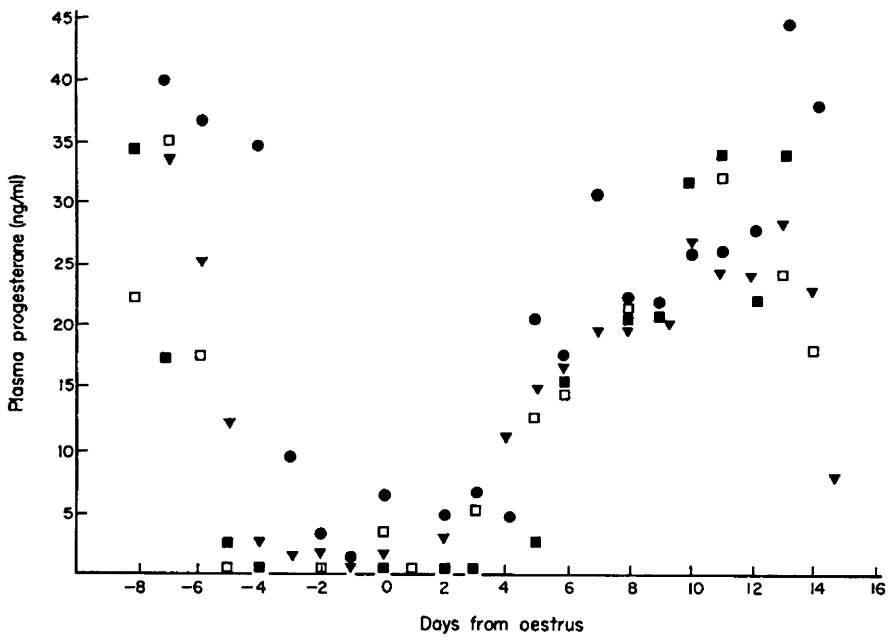
TEXT-FIG. 2. $^3H/^{14}C$ ratios for a pig pregnancy plasma sample through four crystallizations. Vertical bars indicate the range of duplicate determinations.

illustrated in Text-figs. 1 and 2. A replication of the assay on twelve samples of bulked pregnancy plasma gave a mean value of 11·98±0·71 ng with a range of 87 to 130% of the mean.

Recoveries through to the tritiation stage varied from 57 to 75%. It was necessary to determine a standard curve with each set of samples assayed due to deterioration of the tritiated alumina.

For the protein-binding assay, recoveries were assessed by adding [7-^{3}H]progesterone to 1·0-ml samples of pig blood from various reproductive stages before extraction. Recoveries from thirty-six samples were 64±4%. All reported values have been corrected for a recovery of 64%.

Petroleum ether blanks varied from 0 to 0·3 ng. The precision from 0·0 to 4·0 ng, the range of the standard curve most often used was determined for



TEXT-FIG. 3. Progesterone levels in venous plasma during the oestrous cycle. Scatter diagram showing results obtained by different assay methods. □, Pig 555, DID assay; ■, Pig 567, DID assay; ●, Pig 375, CPB assay; ▼, GLC assay (Stabenfeldt *et al.*, 1969).

forty duplicate plasma samples selected at random from five different assays and gave a coefficient of variation of 11·8% (Snedecor, 1956).

Replication on twelve samples of the bulked pregnancy plasma used for DID assay gave a mean value of 14·73±0·92 ng with a range of 68 to 128% of the mean.

Castrate pig plasma was not available but addition of 2·0, 4·0, 8·0 and 16·0 ng progesterone to 1·0-ml aliquots of castrate sheep plasma and assay by the protein-binding method gave values of 104, 105, 96 and 101% of the added values, respectively, after correcting for recoveries and blank values.

Reliability criteria for the oestradiol-17β assay as described by Corker & Exley (1970) were as follows: recovery through the method was 55%±11

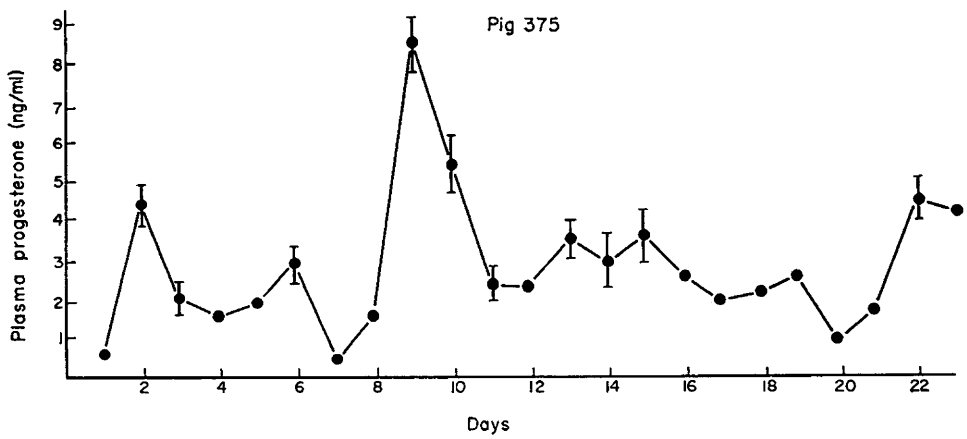
(S.D.) and all values given were corrected for losses of 45%; mean recovery of added amounts of oestradiol-17β (0, 25, 50 and 100 pg in duplicate) to plasma was 95%±18 (S.D.). The precision for a standard curve, range 0 to 100 pg, was ±2·9 pg and the blank value was 5·6 pg±8·8 (S.D.).

Hormone determinations in pig plasma

A scatter diagram for two pig oestrous cycles assayed by DID, one by protein binding, are given in Text-fig. 3. For comparison, the results of Stabenfeldt, Akins, Ewing & Morrissette (1969) using a gas liquid chromatography method are also given.

Progesterone levels for a typical prepubertal period are shown in Text-fig. 4. Duplicate analyses of sixty-six samples from three animals gave a mean progesterone concentration of 2·4±0·2 ng/ml.

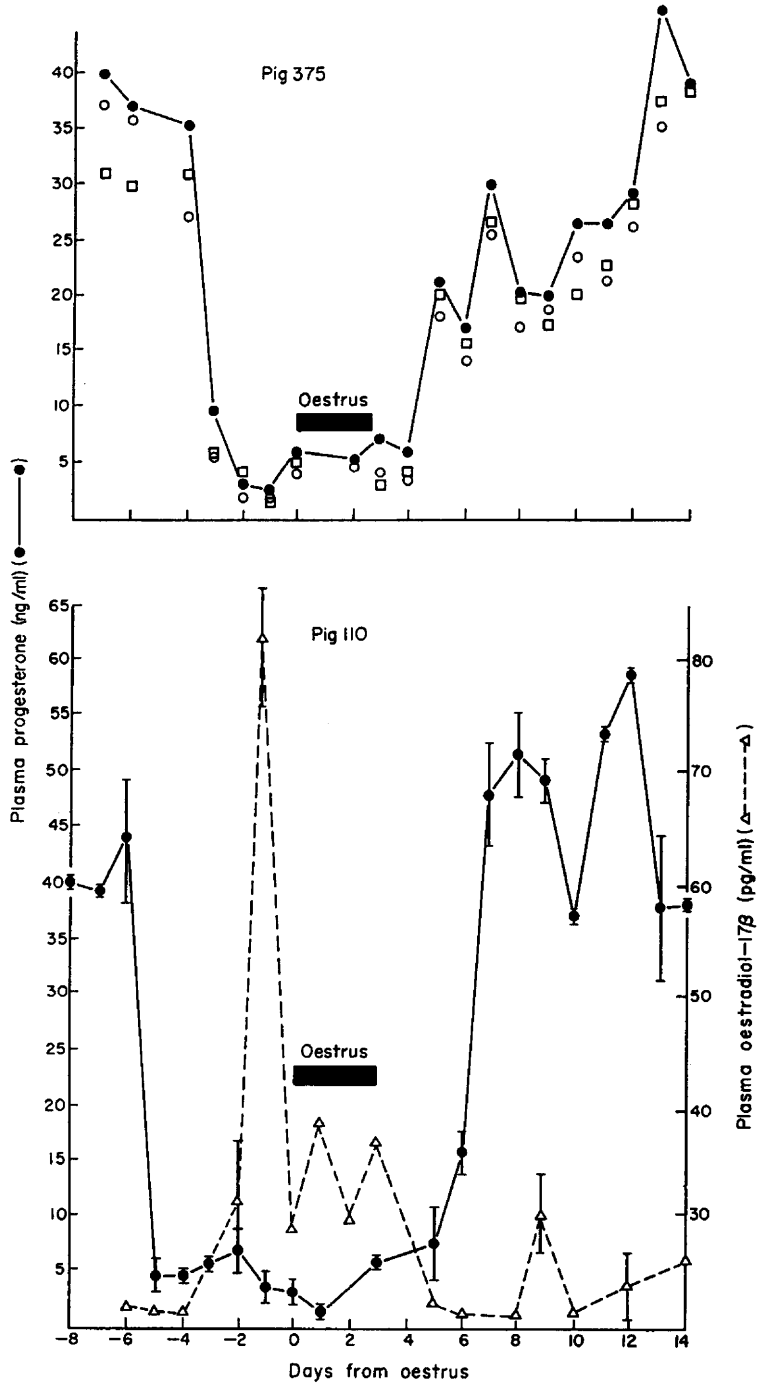
Text-figure 5 shows the progesterone levels through the oestrous cycle of two pigs. The values for Pig 375 were obtained by single protein-binding assays



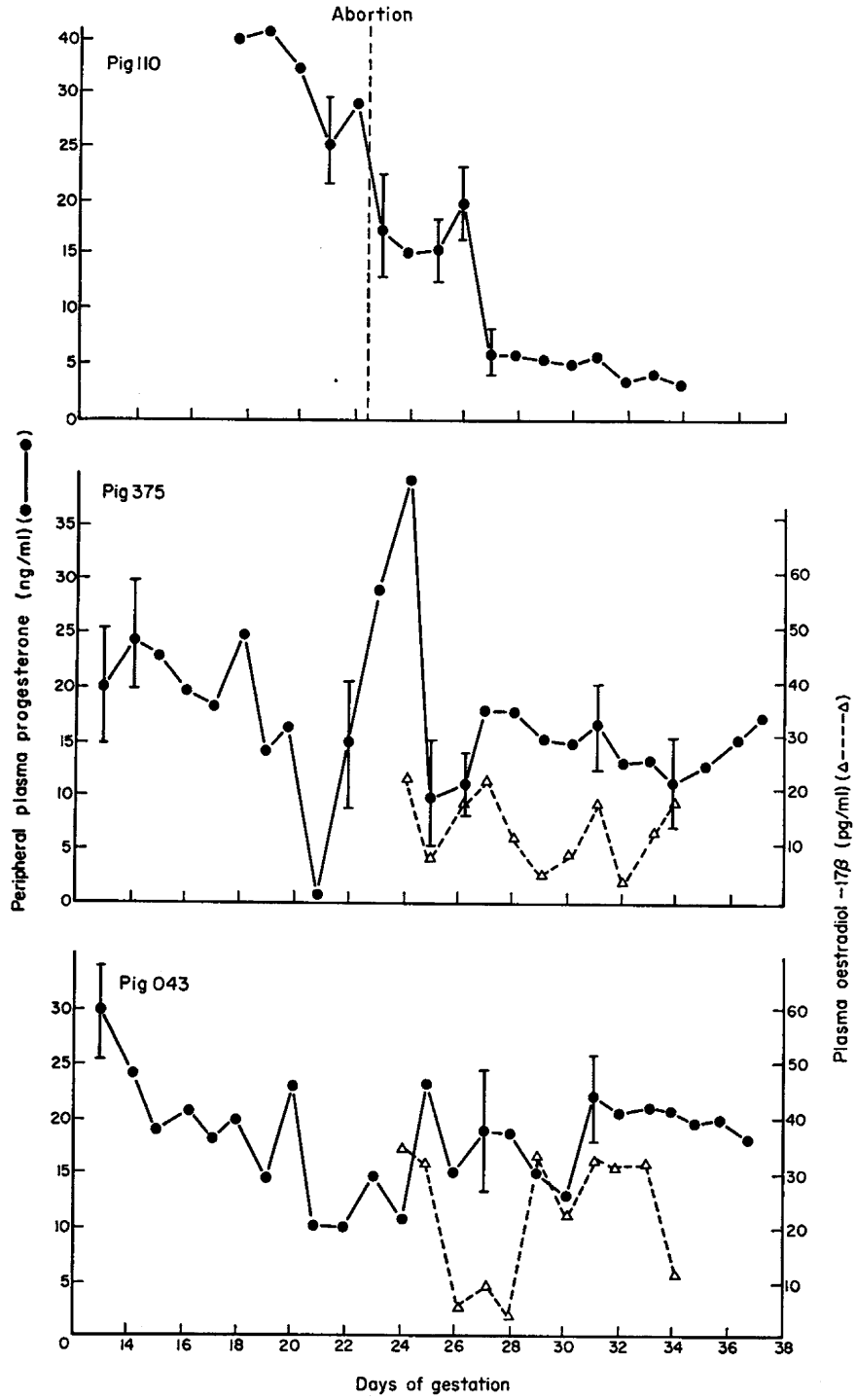
TEXT-FIG. 4. Progesterone levels in venous plasma of a gilt during prepuberty. In Text-figs. 4 to 8, all progesterone levels are means of duplicates. Vertical bars indicate the range of duplicates when they exceed 15%.

repeated three times over a period of 6 weeks. The plasma samples were thawed for aliquot removal and refrozen between each assay. The values for Pig 110 are means of duplicate determinations. In both animals, elevated progesterone levels were apparent until 3 to 5 days before oestrus when a rapid drop occurred to levels ranging from 2 to 5 ng/ml. The levels remained low throughout the pro-oestrous and oestrous phases of the cycle and began to rise near the end of metoestrus. The rate and extent of the rise in circulating levels of progesterone was different in the two animals. The rise in Pig 110 was much steeper and the maximum value of 59 ng/ml was higher than that of Pig 375 and the two animals investigated by the DID technique.

Determination of oestradiol-17β concentrations in Pig 110 (Text-fig. 5) showed a fluctuating level of between 5 and 37 pg/ml until the day preceding the onset of behavioural oestrus when an increase to 85 pg/ml was demonstrated. The peak was of 1 day's duration only and no subsequent rises occurred.



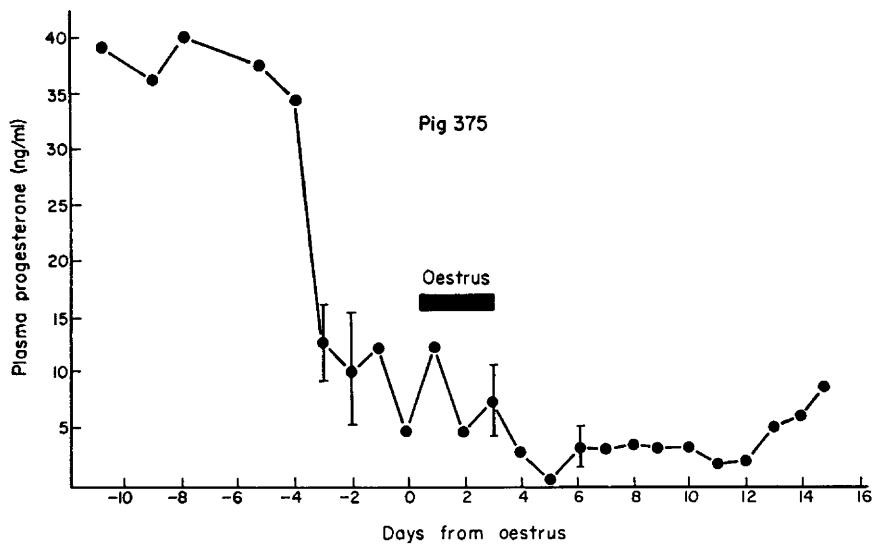
TEXT-FIG. 5. Progesterone and oestradiol-17β levels in venous plasma of gilts during the oestrous cycle.



TEXT-FIG. 6. Progesterone and oestradiol-17β levels in venous blood of gilts during early pregnancy and a spontaneous abortion.

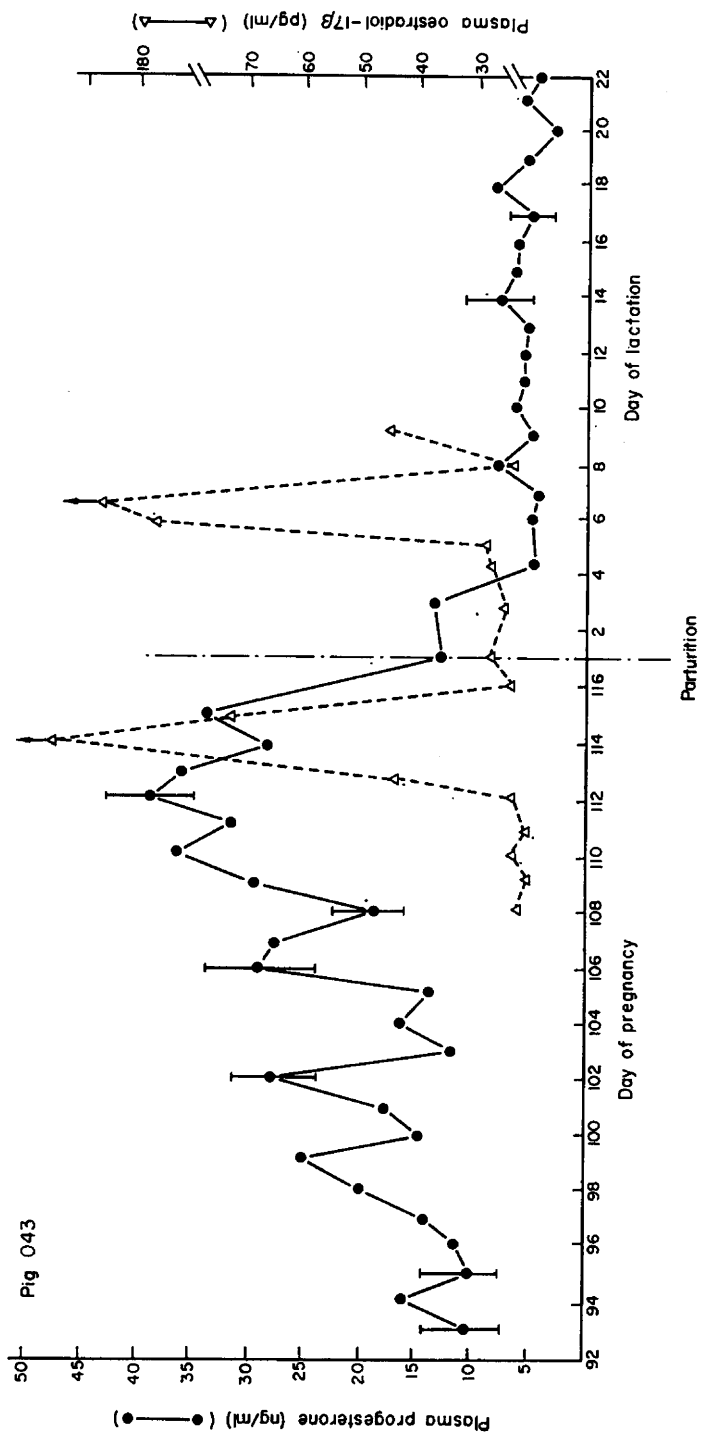
Values of progesterone and oestrogen determinations are shown for two pigs during early gestation (Text-fig. 6). Initial levels of progesterone were similar to those during the luteal phase of the oestrous cycle. A progressive decline occurred until Day 26 to a level lower than that found during the luteal phase of the oestrous cycle. Oestrogen levels remained consistently low throughout. Progesterone levels during early pregnancy for a further pig which spontaneously aborted early in pregnancy are also shown in Text-fig. 6.

Text-figure 7 sets out data collected in retrospect from an animal which mated during the oestrous period shown, did not return to oestrus on Days 23, 24 and 25 and was considered to be pregnant. This animal subsequently showed boar receptivity on Days 79 and 80 of the assumed pregnancy, was remated and became pregnant.



TEXT-FIG. 7. Progesterone levels in venous blood of a gilt assumed to be pregnant but which subsequently returned to oestrus.

Progesterone and oestradiol-17β values for one pig during late gestation, parturition and early lactation are shown in Text-fig. 8. A rise in progesterone concentration began on Day 98 of gestation and reached a plateau by Day 110. A decline commenced before parturition. Analysis of samples withdrawn during parturition gave a concentration of 12·5 ng/ml and a level of around 5 ng/ml was established within 3 days of farrowing. This level remained unchanged throughout the measured period of lactation. A marked elevation in circulating oestradiol-17β levels occurred on Days 4, 3 and 2 before parturition with a peak value occurring on Day 3 which exceeded the 200 pg/ml range of the assay. Levels of approximately 20 pg/ml were recorded on the day of parturition and were maintained until Day 5 of lactation. On Day 6, the concentration was 180 pg/ml and levels on Day 7 again exceeded the range of the assay. Low levels were recorded on Days 8 and 9.



TEXT-FIG. 8. Progesterone and oestradiol-17β levels in venous blood from a gilt during late pregnancy, parturition and early lactation.

DISCUSSION

The use of an indwelling catheter is convenient since blood samples can be taken with minimal disturbance to the animal. Catheters occasionally became blocked for undetermined reasons but several remained patent for periods in excess of 3 months, and one for 8 months.

Erb, Randel & Callahan (1971) have reviewed methods used for the measurement of peripheral progesterone and concluded that agreement between measurement by a DID method, gas liquid chromatography and competitive protein-binding is possible in the sow. The comparisons reported here support this view.

During prepuberty, a mean circulating progesterone level of 2·4 ng/ml plasma was obtained. The origin of this progesterone is not known although plasma progesterone levels exceeding 400 ng/ml in adrenal venous blood were reported in a male pig by Balfour, Comline & Short (1957), who noted that this concentration was some 100 times greater than that measured in peripheral blood collected at the same time. On this basis, peripheral plasma levels of adrenal origin of the order of 4 ng progesterone/ml could be envisaged. However, more recently Heap, Holzbauer & Newport (1966) working with much younger pigs (7 to 13 weeks old) reported their inability to detect progesterone in arterial samples using an assay with a minimum sensitivity of 2 ng/ml.

Patterns of plasma progesterone during the oestrous cycle are in agreement with those reported by other workers (Tillson & Erb, 1967; Stabenfeldt *et al.*, 1969; Edqvist & Lamm, 1971), with the exception that the oestrous values (2 to 5 ng/ml) are somewhat higher than those (1 ng/ml) recorded by Stabenfeldt *et al.* (1969). The results of three assays for Pig 375 carried out at different times suggest that repeated thawing and refreezing of plasma may have a small but detrimental effect on progesterone. The difference between the data for Pig 110 and that recorded for the oestrous cycles of other animals is striking, progesterone levels during the luteal phase being significantly higher. Levels during early pregnancy were also higher than those found in other animals and the animal had a spontaneous abortion. Progesterone levels during the period of abortion indicate that the cause was not failure of the corpora lutea since the progesterone concentration remained high for 4 to 5 days after the event. It is interesting to note that the low fertility found in sheep induced to breed out of season may be associated with elevated levels of progesterone (Haynes & Lamming, 1971).

The overall result of this study, for animals in early pregnancy, supports the work of Tillson, Erb & Niswender (1970) which showed a gradually declining level of circulating progesterone with advancing pregnancy.

During late gestation, limited available information suggests a decline in circulating levels of plasma progesterone in the pig approaching parturition (Short, 1960; Masuda, Anderson, Henricks & Melampy, 1967). The present data demonstrate a rising level from Day 98 of gestation to about Day 110, with a decline occurring 2 days before parturition. Edgerton & Erb (personal communication) have shown that the total excretion of progesterone metabolites more than doubled from Days 61 to 103 of pregnancy and declined only slightly by Day 110. Similar increases in plasma progesterone concentration in late

pregnancy have been noted in the cow (Donaldson, Bassett & Thorburn, 1970) and in the ewe (Bassett *et al.*, 1969).

There is substantial evidence that functional corpora lutea are absent from the ovaries of lactating sows (cf. Crighton & Lamming, 1969). The consistently low levels of circulating progesterone reported in the present study support these observations.

The daily progesterone levels for Pig 110 (Text-fig. 7) illustrate the potential value of progesterone determinations as a diagnostic test in the pig, and indicate that the oestrous period at which the animal mated was probably anovulatory.

The recorded rise in oestradiol-17β immediately before oestrus is in accord with determinations in the sheep (Norman, Eleftheriou, Spies & Hoppe, 1968; Shutt, 1969) which indicate peak concentrations of oestrogen immediately before or during early oestrus. The rise in oestradiol-17β also correlates well with evidence for a peak of urinary oestrogen approximately at the onset of oestrus in the pig (Raeside, 1963a, b; Liptrap & Raeside, 1966). These workers state that the renal elimination of oestrogen is almost completely in the form of oestrone. The identification of oestradiol-17β in the circulating plasma of the pig gives support to suggestions of a peripheral transformation of oestrogens in the pig (Lunaas, 1963).

Evidence is available for a transient rise in urinary oestrogen between Day 24 and Day 32 of gestation in the pig (Lunaas, 1962, 1963; Edgerton & Erb, personal communication). Attempts to demonstrate a concurrent rise in circulating levels of oestradiol-17β in the present study were not successful. However, Drane & Saba (1967) measured total circulating oestrogens using a bioassay technique and stated that the bound oestrogens were elevated at Day 25 of gestation compared with Day 60 but free oestrogen remained low until a month before parturition.

Evidence from a number of sources suggests that the 24-hr level of urinary oestrogen rises steeply from about the 10th week of gestation to reach milligram amounts immediately before parturition (Velle, 1958; Raeside, 1963b; Fèvre, Léglise & Rombauts, 1968). Levels showed a 100-fold reduction in oestrone excretion after parturition and levels in lactation appeared to be uniformly low. The plasma oestradiol-17β pattern before parturition is decidedly different to that recorded by urine analysis. An increase was only detected in circulating oestradiol-17β 4, 3 and 2 days before parturition and a lower level was again established at the onset of parturition. A transient peak of plasma oestrogen also occurs on the day before parturition in the ewe (Challis, 1971). Again, this bears little relationship to oestrogen excretion as a rising level of urinary oestrogen with advancing pregnancy which is more gradual has been reported by Fèvre & Rombauts (1966). The fall in oestrogen concentration occurs much closer to the time of parturition in the sheep than is indicated in the pig. The relative infrequency of sampling in the experiments reported here, however, precludes any definitive conclusions as to the relationship of oestradiol-17β levels and the onset of parturition in the pig. Furthermore, the assay used specifically measures oestradiol-17β which may be of minor significance in the pig in view of the high levels of urinary oestrone.

The unexpectedly high concentration of plasma oestradiol-17β after parturition may be in some way responsible for the hitherto unexplained phenomenon of a *post-partum* anovulatory oestrus in sows (Warnick, Casida & Grummer, 1950; Burger, 1952). Crighton & Lamming (1969) have shown that follicular growth and uterine development are suppressed during lactation and placental oestrogen is no longer present. The source of this plasma oestrogen is therefore, not apparent and further studies are in progress to determine its origin.

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