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Plasma Estrogen, Progesterone and Luteinizing Hormone Levels During the Estrous Cycle in Pigs[1]

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Plasma estrogen, progesterone and luteinizing hormone (LH) concentrations were determined during the estrous cycle in six pigs and throughout the day on the first day of estrus (Day 0) in four pigs. Fifty-seven blood samples were collected during the cycle and 16 samples on the day of estrus. The mean plasma estrogen level was low (<20 pg/ml/animal) during the cycle until Day 16 or 17 when it began to increase reaching a maximum level (>50 pg/ml/animal) one or two days prior to estrus. The mean progesterone level increased rapidly from 5.1 to 24.2 ng/ml/animal between Days 2 and 6, and continued to increase to reach a maximum level (33.2 ng/ml/animal) by Day 12. The mean estrogen level began to increase only after the progesterone level began to fall. The mean LH level was 2.5 ng/ml/animal on Day 0, then decreased below 1 ng/ml/animal until the next estrus. At estrus, the LH level increased to a high level (>5 ng/ml/animal), while the estrogen level was high (>20 pg/ml/animal) and progesterone remained below 1 ng/ml/animal.

Knowledge of the plasma levels of a hormone is required for an understanding of the dynamics of its synthesis and secretion by the endocrine gland. Little is known of plasma estrogen levels in most species including the pig. From studies of sow urine, it was found that estrone was the preponderant estrogen. While some estradiol-17β was detected (Velle, 1958; Terqui *et al.*, 1968) during the cycle, a rise in urinary estrone occurred only at estrus (Lunass, 1962; Raeside, 1963; Liptrap and Raeside, 1966). If the sow was pregnant, urinary estrone rose approximately 3 weeks after mating (Lunass, 1962). In the ovary, the preponderant estrogen was estradiol-17β. Some estrone was present (Westerfeld *et al.*, 1938). With the development of specific, sensitive radioimmunoassays, it is now feasible to measure plasma estrogen concentrations (Abraham, 1968; Scaramuzzi *et al.*, 1970).

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Radioimmunoassays for porcine luteinizing hormone (LH) have been reported for the determination of serum LH concentrations during the estrous cycle of gilts (Niswender *et al.*, 1970; Rayford *et al.*, 1971). Using gas–liquid chromatography (Stabenfeldt *et al.*, 1969), and the competitive protein binding assay (Tillson *et al.*, 1970), progesterone levels have been determined during the estrous cycle. The purpose of this study was to determine the concentrations of estrogen, progesterone and LH in peripheral plasma during the estrous cycle of the pig on an every other day basis and throughout the day on the first day of estrus.

MATERIALS AND METHODS

Animals. Ten Duroc gilts, 8 to 10 months of age, were used in this experiment. All gilts had exhibited 2 estrous cycles of normal length (18 to 23 days) prior to the experiment. Incidence of estrus was determined by placing a vasectomized boar with the gilts at approximately 8 AM each day. Six gilts were bled once per day (8 AM) every 2 days during a complete estrous cycle. Four other gilts were bled more often on the first day of estrus (Day 0). Blood was collected at 8 AM, 11 AM, 2 PM and 5 PM. These gilts were checked

each day at 8 AM and 5 PM for estrus. The four gilts were not in estrus at 5 PM on the day preceding the day of estrus. Approximately 30 ml of blood was drawn into a heparinized syringe from the anterior vena cava as described by Carle and De Whirst (1942). The blood was centrifuged within 1 hr after bleeding at 4C and plasma harvested and stored at −20C until the assays were performed.

Plasma estrogen assay. Samples were assayed in duplicate for total estrogen using 2- to 4-ml aliquots. After adding 1 drop of ammonium hydroxide and extracting the sample with 4 ml of fresh diethyl ether 2 times, the ether was transferred to a disposable glass tube and evaporated under a stream of air leaving a dry extract. The extract was incubated at 2–4C in 0.1 M phosphate buffer containing 0.1% gelatin, [6,7-$^3H_2$] estradiol-17β, 7-^{3}H (0.009 μCi, spc act of 40 Ci/mmole) and antisera. A dilution of 1 part antisera to 15,000 parts buffer was used and it bound 55–65% of the [^{3}H] estradiol-17β in a tube containing no unlabeled estradiol-17β. The unbound estrogen was removed from the solution by adding 1 ml of a dextran—charcoal solution (250 mg charcoal, 25 mg dextran T-80, Sigma Chem. Co.) and centrifuged at 4C. Radioactivity was counted in a Nuclear–Chicago liquid scintillation counter. A set of tubes (in duplicate) containing 10, 20, 50, 100, 200 and 400 pg of estradiol-17β (Mann Biochemical Co.) per tube was included in all assays. The values obtained from this set were used to construct a standard curve.

Two pairs of tubes containing 4 ml of plasma each from a 3-month-old castrated male pig were included in each assay. The plasma in one pair of tubes was used to determine a blank value for the assay. The plasma in the second pair of tubes received 50 pg of estradiol-17β to obtain an estimate of the recovery of the authentic estrogen. Plasma in a third set of four tubes received 10,000 cpm of [6,7-$^3H_2$] estradiol-17β to obtain an estimate of recovery of labeled estradiol-17β. The blank value ranged from 16 to 24 pg for a 2-ml sample of plasma. The mean blank value in each assay was subtracted from each sample value. Following this adjustment, the mean per cent recovery of the labeled estrogen was used to correct the sample value for procedural losses. The recovery of [^{3}H] estradiol-17β ranged between 90 and 96% for all assays and differed less than 2% between the 2 tubes in an assay. The amount of variation between duplicate samples analyzed in the same assay was determined. The coefficient of variation was 16.6% (n = 68).

Before analyzing plasma samples on a routine basis, 25, 50, 100 and 200 pg of a standard preparation of estradiol-17β in ethanol were added to 0.25, 0.5, 1.0 and 2.0 ml of plasma from an ovariectomized gilt, respectively. After extraction as discussed previously, the extracts were assayed against aliquots from the same standard preparation. The standards were pipetted directly into tubes and the ethanol evaporated. The results are shown in Fig. 1.

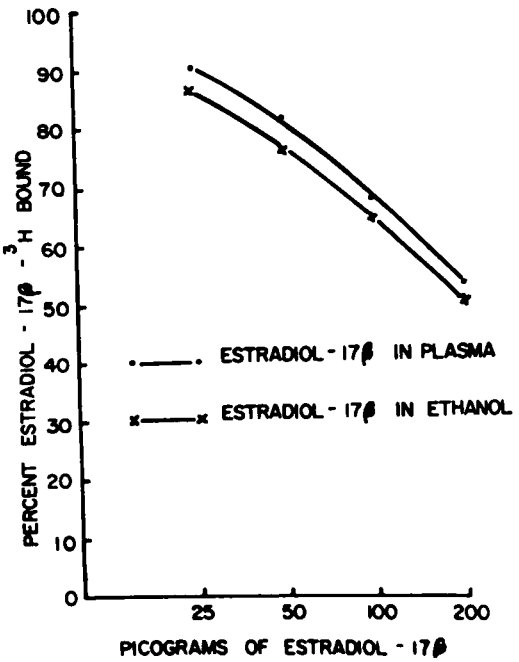

FIG. 1. Effect of quantity of estradiol-17β in ethanol and in porcine plasma on percent of [^{3}H] estradiol-17β bound to antisera. Each point is the mean of duplicate determinations.

Aliquots consisting of 0.25, 0.5, 1.0 and 2.0 ml of plasma sample which was collected from a gilt on day 16 of the cycle were assayed in duplicate. The concentrations of estrogen per ml were 12, 16, 15 and 18 for the 4 aliquots. The antisera was kindly supplied by Dr. B. V. Caldwell of the Yale School of Medicine. The preparation of the antisera has been described by Tillson *et al.* (1971).

Plasma progesterone assay. The competitive protein assay (Murphy, 1967; Neill *et al.*, 1969) was used to quantitate progesterone concentration in the plasma samples.

The samples were analyzed against aliquots of a standard preparation of pig plasma containing a known quantity of authentic progesterone. This was prepared by adding 1 ml of a stock solution containing 1 mg of progesterone/ml of 95% ethanol to 100 ml of ovariectomized pig plasma. Before adding the progesterone (Mann Biochemical Co.), the plasma was assayed for endogenous progesterone. None was detected. A standard solution of progesterone in 95%

ethanol having the same progesterone concentration (10 ng/ml) as the plasma standard was also prepared.

The assay design was a 4-point parallel line assay (Finney, 1964). Three pairs of centrifuge tubes receiving 0.1, 0.3, or 0.9 ml of the standard plasma preparation corresponding to 1, 3, or 9 ng of progesterone were prepared. Duplicate tubes containing 2 quantities of sample plasma were also prepared. The quantities were either 0.1 and 0.3 ml or 0.3 and 0.9 ml depending upon the expected quantity of progesterone present in the sample. The 2 responses from the sample were compared with the 2 responses from the standard preparation which were similar. The log-dose response curve for the standard preparation was linear from 1 to 9 ng.

All tubes were extracted with 10 volumes of petroleum ether (30–60C). The ether was pipetted into disposable glass tubes and evaporated. One ml of binding protein solution consisting of 2 μCi (14 ng) of [1,2-$^3H_2$] corticosterone (B-^{3}H) and 0.5 ml of male dog plasma in 100 ml of distilled water was added to each tube and incubated for 30 min in an ice bath. The unbound progesterone was precipitated with Florisil, and an aliquot of the supernatant liquid was counted by liquid scintillation. Using 2 responses from the standard preparation and those from the samples,

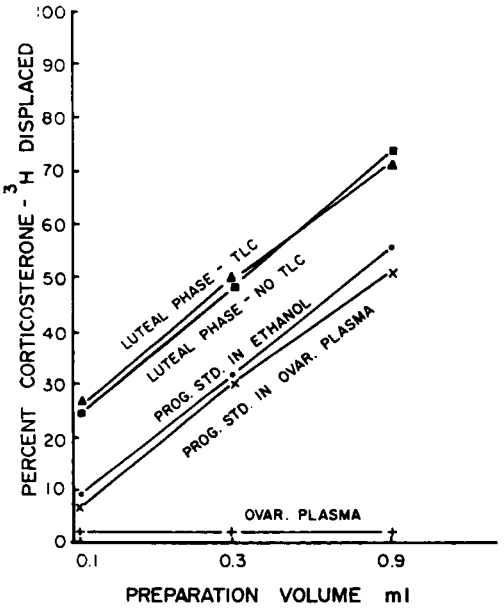

FIG. 2. Effect of volume of various preparations in percent of [^{3}H] corticosterone displaced from binding protein. The standard preparation consisted of 0.1, 0.3 and 0.9 ml of ovariectomized plasma containing 1, 3 and 9 ng of progesterone, respectively. Each point is the mean of duplicate determinations.

a relative potency was calculated. Each assay was also evaluated for parallelism and index of precision according to Emmens (1948). Parallel line assay statistics may not be valid for competitive protein binding assays (Rodbard *et al.*, 1968). Homogeneity for variance may not occur in the dose response curves for this assay. This possibility was tested by determining the response variances for 2 tubes at each of 4 concentrations, 1, 3, 9, and 25 ng of progesterone, in 4 assays. The variances for the 4 concentrations were 3.2, 1.5, 0.7 and 1.2, respectively. Using Bartlett's test for homogeneity of variance, the variances were homogeneous ($p > 0.05$).

The possibility that, in addition to progesterone, petroleum ether removes other steroids from pig plasma that have an affinity for the binding protein was examined to determine if a thin layer chromatography (TLC) step was necessary as found by Neill *et al.* (1969) in a study of human plasma.

In 11 assays, luteal pig plasma and the standard preparation were submitted to the above protocol with and without a chromatographic step included. The values for progesterone (TLC *vs.* no TLC) in 3 of these assays were 21.1 *vs.* 20.5, 22.1 *vs.* 24.1, and 25.8 *vs.* 22.9. The mean values ± SD for the 11 assays were 21.3 ± 2.0 *vs.* 22.8 ± 2.3 ng/ml.

The validity of the assay procedure was examined. Three quantities, 0.1, 0.3, and 0.9 ml, of each of 4 preparations were tested for ability to give a linear response with respect to the 3 doses and for parallelism between the preparations. The results are shown in Fig. 2. The progesterone standard in ethanol, progesterone standard in ovariectomized plasma and luteal phase plasma behaved similarly in the assay. There was little difference in the response curves whether or not the luteal phase plasma was chromatographed. The response lines were linear and parallel ($p < 0.01$). There was slight displacement of the B-^{3}H from the binding protein by ovariectomized plasma (see Fig. 2).

Plasma LH assay. Plasma porcine LH was assayed by a radioimmunoassay as adapted from the method of Niswender *et al.* (1970) who described its specificity and sensitivity. Antisera for porcine LH (LER-778-4) was kindly supplied by Dr. G. Niswender, Univ. of Michigan. The antisera was diluted 1:32,000 with buffer for use in this study. Porcine LH (LER-786-4), having an LH activity of 0.65 NIH -LH -SI units/mg was used for radioiodination with sodium ^{131}I iodide (Iso-Serve Div. of Cambridge Nuclear Corp.) and for preparation of the standard concentrations.

When the antiporcine LH serum was diluted 1:32,000, approximately 45% of the PLH-^{131}I was bound to the antibody in those tubes containing no competing unlabeled hormone. The radioactivity bound to antibody in the buffer control tubes was assigned the value of 100%. Figure 3 depicts dose

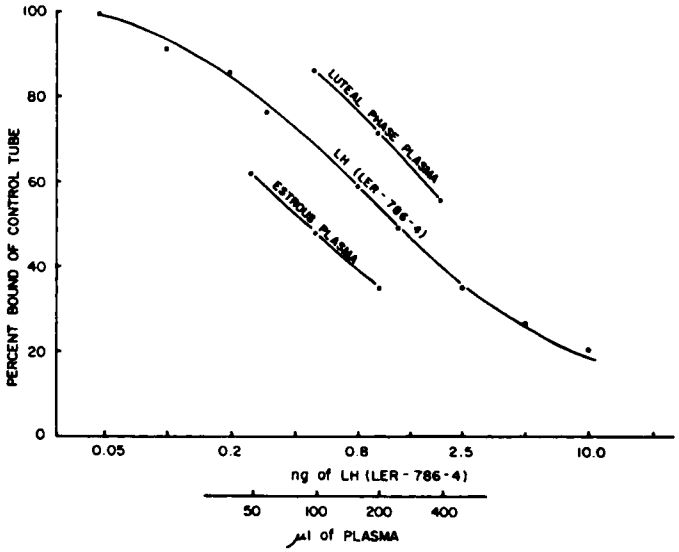

FIG. 3. Standard preparation of LH and porcine plasma displacement curves. Each point is the mean of triplicate determinations.

response curves which show the decreased quantity of PLH-^{131}I bound to the antibody which occurred with increasing quantities of purified porcine LH (LER-786-4) and increasing volumes of pig plasma collected at estrus and during the luteal phase. The LH was kindly supplied by Dr. L. E. Reichert, Emory University. Antirabbit gamma globulin prepared in this laboratory was used to precipitate the original antisera.

RESULTS

The individual levels of total estrogen, progesterone and LH found in plasma samples which were collected from six gilts throughout the estrous cycle are depicted in Figs. 4 and 5. There were variations in the length of the cycle (1 pig, 19 days; 2 pigs, 20 days; and 3 pigs, 21 days). As might be expected, there was considerable variation in the patterns of hormone levels among the gilts.

In four of the six gilts, LH was at a maximum level on the first day of estrus (Day 0). The levels in the six gilts ranged from 4.6 to 0.6 ng LER 786-4/ml. Since blood was obtained at 8 AM and the last observation for estrus was made at 5 PM the previous evening, it is possible that the LH level had begun to fall from the maximum level by the time the sample on Day 0 was collected. None of the gilts were displaying signs of estrus which could be detected by a vasectomized boar at the 5 PM check prior to estrus. During the remainder of the cycle, the level remained below 1.0 ng/ml in all but one animal. This animal exhibited a level above 2 ng/ml on Days 6, 16 and 18 of the cycle. The mean LH level rose slightly at the same time that the mean progesterone level began its precipitous fall.

Progesterone levels began to increase rapidly within 2 days after estrus. By Day 6 of the cycle the mean level was in excess of 24 ng/ml. The maximum level, ranging from 28 to 48 ng/ml, was reached on Days 7, 11, 11, 12, 12 and 13 in the 6 gilts. After remaining on a plateau for 2–3 days, the level declined rapidly in all gilts. The decline from the steady high level to less than 3 ng/ml occurred within 2 days in all gilts except one. In this animal the level decreased to 8 ng/ml within 2 days. There was a period of 4–6 days prior to estrus during which the level was less than 1 ng/ml.

The levels of estrogen in pig plasma were in the range of 10–30 pg/ml throughout

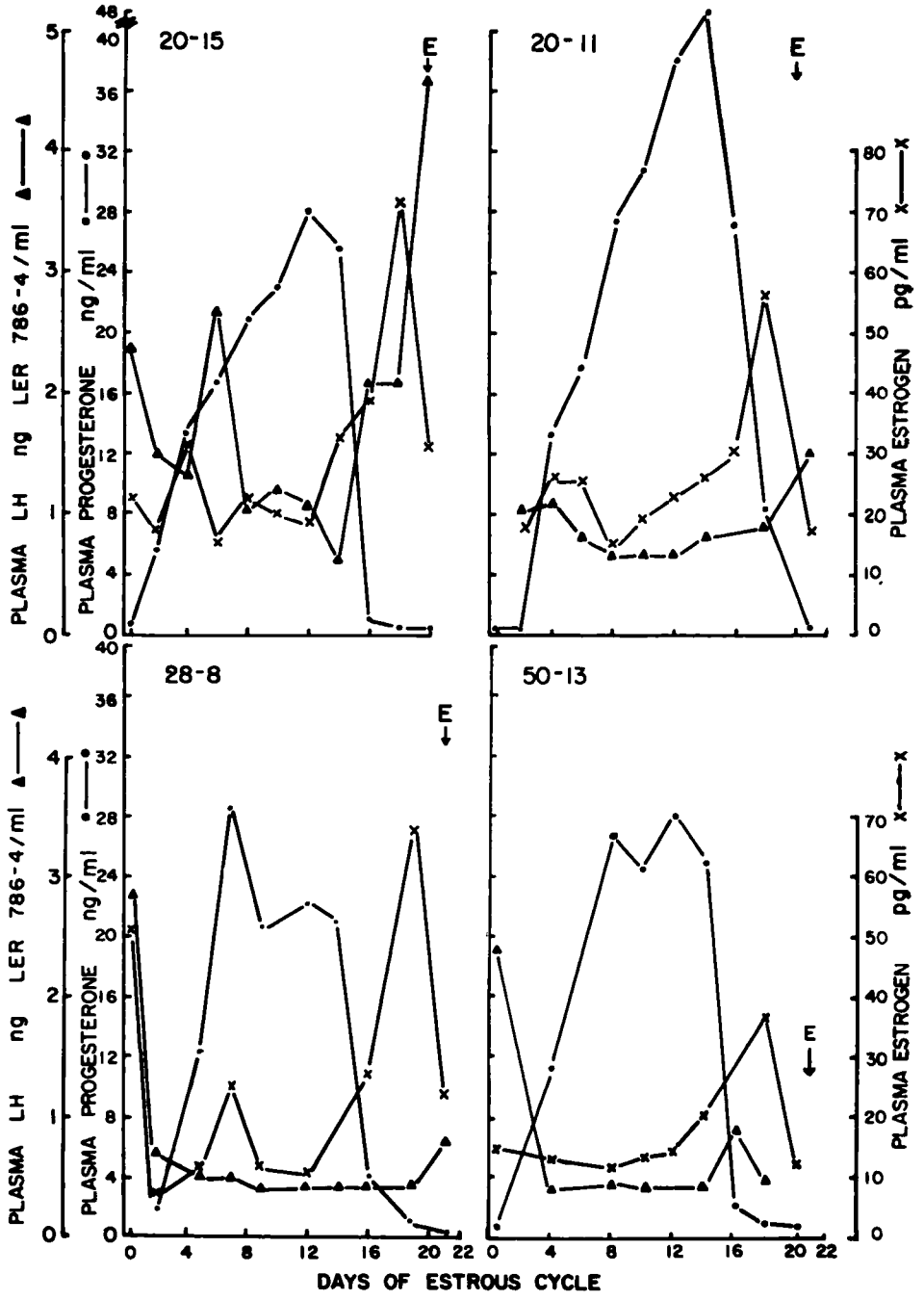

FIG. 4. Plasma estrogen, progesterone and LH concentrations in individual gilts (20-15, 20-11, 28-8 and 50-13) during estrous cycle. Each point is the mean of duplicate determinations.

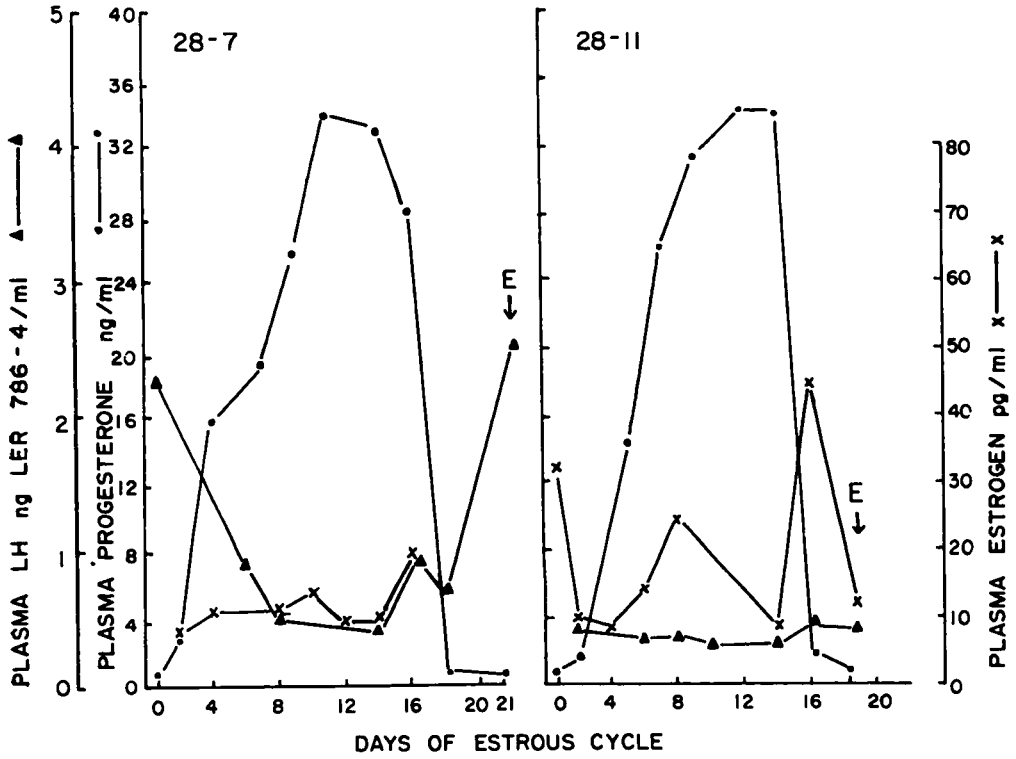

FIG. 5. Plasma estrogen, progesterone and LH concentrations in individual gilts (28-7 and 28-11) during estrous cycle. Each point is the mean of duplicate determinations.

much of the cycle except for the day prior to estrus when there was an increase in concentration to 60–70 pg/ml. There appeared to be a cyclic change in the level in which a small increase to 20–30 pg/ml occurred on Days 4 or 5 of the cycle. The level then declined to 10–20 pg/ml until Days 14 or 15 when a slow increase began until the second day prior to estrus. Two days prior to estrus, the level increased sharply. On the day of estrus, the level declined, but was quite variable ranging from 12 to 86 pg/ml. The increase in estrogen level just prior to estrus was detected in 5 of the 6 animals. The estrogen concentration reached a maximum level 48 hr prior to the time the maximum LH concentration occurred which was on the first day of estrus.

The levels of the 3 hormones in plasma at 4 definite times on the first day of estrus in 3 pigs are given in Table 1. The LH levels reached a peak at 11 AM in 1 gilt, at 5 PM in 2 gilts and at 2 PM in 1 gilt for which the data are not shown. The maximum levels were 8.9, 5.4, 11.8 and 1.6 ng/ml, respectively. As indicated in Table 1, the level of LH was increasing throughout the day of estrus in those pigs having a peak level at 5 PM. The estrogen levels were higher in the pigs (30–70 pg/ml) having a maximum LH level later in the day than in the pig in which plasma LH reached a peak at 11 AM (9–28 pg/ml). The latter animal may have been in estrus longer than the other animals when blood collection began. Progesterone values were near the nondetectable level in all gilts throughout the first day of estrus.

DISCUSSION

Since each plasma sample was assayed for estrogen, progesterone and LH, some conclusions can be made regarding the temporal

TABLE 1

PERIPHERAL PLASMA CONCENTRATIONS OF PROGESTERONE, TOTAL ESTROGEN AND LUTEINIZING HORMONE ON FIRST DAY OF ESTRUS[a]

	First day of estrus			
	8 AM	11 AM	2 PM	5 PM
Pig 50–13				
Prog (ng/ml)	0.5	0.5	0.5	0.4
Estrogen (pg/ml)	50	35	43	42
LH (ng LER786-4/ml)	1.0	1.4	4.7	5.4
Pig 57–12				
Prog (ng/ml)	0.3	0.4	0.4	0.7
Estrogen (pg/ml)	28	73	54	41
LH (ng LER 786-4/ml)	2.7	3.3	8.5	11.8
Pig 58–7				
Prog (ng/ml)	0.5	0.6	0.8	0.7
Estrogen (pg/ml)	28	9	14	22
LH (ng LER 786-4/ml)	4.8	8.9	3.4	4.8

[a] All animals were in estrus at 8 AM but not in estrus at 5 PM on the preceding day.

relationships among the plasma concentrations during the estrous cycle. Of interest also, is whether or not plasma levels fit the concepts of secretion of these hormones that have been suggested by other types of experiments.

Plasma LH levels remained low but not entirely absent throughout the cycle, except on the first day of estrus. This result may have been due to the suppressing action of the rapidly rising level of progesterone for the first 14–15 days of the cycle. The mean level appeared to rise slightly from 0.5 ng/ml to nearly 1.0 ng/ml after the rapid decline in progesterone level on Days 14–15. The mean concentrations reported in this study are very similar to those reported by Niswender *et al.* (1970) who also determined LH levels during the estrous cycle. While not differing greatly from the mean concentrations reported for the cycle by Tillson *et al.* (1970) and Rayford (1971), the levels obtained at times other than on day 0 in this study were lower. The levels at estrus were much lower than those found in the cow or the ewe (Henricks *et al.*, 1970; Niswender *et al.*, 1968). The levels on Day 0 indicated that the LH peak occurred in some pigs several hours after the onset of estrus.

Liptrap and Raeside (1966) reported that urinary estrogens in the sow reached a peak about 40 hr prior to ovulation which would be about the time of onset of estrus. Their data indicated that levels began to rise several days prior to that time. The plasma levels obtained in this study followed a similar pattern. Even though the levels were highest on the day of estrus, they were high on the two days prior to estrus. The pattern of plasma estrogen levels supports the concept that estrogen facilitates the release of LH (Sawyer, 1964).

A significant finding was the fact that plasma estrogen concentration did not begin to rise rapidly until the day plasma progesterone concentration began to fall. This phenomenon occurred in every animal. There was a minor peak of the estrogen level between Days 4 and 8 in all 6 pigs. Another report indicates that following estrus, follicle size increases for about the first 8 days followed by little change in follicular size until the time near estrus (Robertson, 1969).

The levels of plasma progesterone obtained in this study, in which the TLC step was omitted from the assay, were in the same range of values and followed the same pattern during the cycle as reported by Stabenfeldt *et al.* (1969) using a gas–liquid chromatographic method and Tillson *et al.* (1970) using both the protein binding procedure and the double isotope derivative procedure. Thus, it appears that other progestins contributed insignificantly to the progestin level in the pig. Although the mean level of progesterone in the 6 pigs did not reach a peak until Day 12, the progesterone level was approximately two-thirds of the maximum level by Days 6–8 of the

cycle. The progesterone concentration of ovarian plasma and luteal tissue reached a maximum level by Day 8 of the cycle (Masuda *et al.*, 1967). The rapid decline in levels on Days 14–16 agree with those reported previously (Stabenfeldt *et al.*, 1969; Tillson *et al.*, 1970) and indicate a rapid regression of the corpora lutea. The values which were found in sequential blood samples collected on Day 0 of the cycle suggest that there is no preovulatory release of progesterone from the ovary as previously shown in the monkey (Johannson *et al.*, 1968).

These findings on the concurrent analysis of total estrogen, progesterone and LH in peripheral plasma point to the need for additional studies to determine: the individual estrogens and concentration in plasma during the cycle, hourly monitoring of hormone levels just prior to and after the onset of estrus and comparable data on some other days of the cycle, and the hormone levels during pregnancy in order to contrast those concentration patterns with those of the estrous cycle. A study is being completed on the plasma hormone levels during pregnancy.

ACKNOWLEDGMENTS

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