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Diurnal Steroid Patterns During Gestation in the Rhesus Macaque: Onset, Daily Variation, and the Effects of Dexamethasone Treatment

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ABSTRACT

Serum samples for cortisol, estradiol-17β (E_2), and progesterone analysis were obtained twice daily or more frequently to determine the onset of diurnal steroid patterns in the maternal circulation of pregnant rhesus monkeys. The expected a.m./p.m. fluctuations in serum cortisol (31.6/22.9 μg/dl) were evident from Day 25, whereas nocturnal elevations in progesterone (9.62/16.31 ng/ml) were demonstrable on Day 30, and diurnal increments in E_2 (283/138 pg/ml), coincident with those of cortisol, appeared about Day 40. Microscopic examination of fetal adrenals from rhesus embryos (ages 30 to 50 days) indicated that the gland differentiates and undergoes extensive development when diurnal E_2 release first appears.

Administration of 1.0 mg of dexamethasone twice daily to pregnant animals on Days 124 to 130 was accompanied by 1) complete suppression of cortisol release, 2) loss of the diurnal E_2 pattern, which was associated with a marked reduction in serum E_2, and 3) eventual loss of the nocturnal elevation in serum progesterone without altering its basal levels. Similar treatment earlier in gestation reduced serum levels of both cortisol and E_2 without influencing the diurnal serum patterns of these hormones or progesterone. These results support the concept that maternal estrogen levels depend on a functional fetal adrenal and further suggest the hypothesis that the inverse patterns in serum cortisol and progesterone are related to altered binding of progesterone to serum proteins as adrenal activity varies throughout the day.

Further evidence supporting the hypothesis of steroid-serum protein interaction was the appearance of nocturnal increments in serum progesterone in ovariectomized animals treated with Silastic capsules containing progesterone and the absence of such increments in similarly treated ovairectomized animals after pituitary stalk section to suppress circadian adrenal activity. It is now clear that diurnal rhythms in serum E_2 and progesterone as well as cortisol are present during most of gestation in the rhesus macaque, but the physiological importance of such rhythms remains to be elucidated.

INTRODUCTION

Daily concentrations of progesterone and estrogen in the maternal circulation during gestation in the rhesus macaque have been well documented (Neill et al., 1969; Atkinson et al., 1975). Recently, other investigators have described rhythmic variations during the last few weeks of primate gestation in the serum concentrations of cortisol and progesterone (Challis et al., 1980; Sholl et al., 1979a), as well as estrogen (Townsley et al., 1973; Sholl et al., 1979b; Patrick et al., 1980). The occurrence of such diurnal steroid patterns in the maternal circulation at earlier stages of gestation, their source, the factors regulating their appearance, and their importance in the gestational economy need further research.

We designed studies 1) to determine the timing of onset of the diurnal patterns in maternal progesterone and estradiol-17β (E_2) concentrations during gestation; 2) to determine the time course of each hormone throughout the day at different stages of gestation; and 3) to observe the effects of adrenal suppression

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on their characteristic fluctuations. Furthermore, since diurnal variations in the levels of progesterone have been observed during the luteal phase in nonpregnant macaques (Spies et al., 1974), we examined ovariectomized monkeys with and without diurnal adrenal function, treated with Silastic capsules containing progesterone, to determine if similar fluctuations would occur when the hormone was provided from a relatively constant release source in another model system.

MATERIALS AND METHODS

Female rhesus macaques *(Macaca mulatta)* were exposed to males for 3 days during the periovulatory period, and pregnancy was assessed with agglutination inhibition test kits for urinary gonadotropins (Hodgen and Ross, 1974) on Days 18 to 21 (Day 1 of pregnancy was arbitrarily selected as Day 2 of male exposure). These studies were carried out over a 2 year period in 32 pregnant and 14 ovariectomized females weighing 4.4 to 7.2 kg and maintained in individual cages at either the Oregon (ORPRC) or California (CPRC) Primate Research Centers in a controlled environment (23°C, 45% humidity) with a 12L:12D photoperiod. They were given Purina monkey chow, and water was available ad libitum. Blood samples were obtained within 3 min of the initial approach by venipuncture either in the animal's own squeeze cage or after transfer to a free-standing squeeze cage. For those samples obtained during the lights-off period, the lights were briefly turned on to facilitate animal handling.

In the initial descriptive experiments on diurnal fluctuations in early gestation, 2.0 ml of blood were obtained from six females (four from the ORPRC and two from CPRC) at 0730 and 1530 h each day beginning on Day 25 of gestation and ending on Day 60. Ten females at the CPRC were each bled twice daily (2.0 ml) for a single 5 day period, at approximately 10 day intervals, to confirm the presence of the diurnal steroid rhythms from Days 60 to 150.

Since results based on twice daily samples could only suggest the presence of diurnal hormone rhythms, eight additional females (six from the ORPRC and two from the CPRC) were bled at 3 h intervals (1.5 ml) for 48 h on Days 28 to 31 and divided into two groups for subsequent bleeding on Days 47 to 51 (n = 4) and Days 107 to 116 (n = 4).

In an assessment of the relationships between adrenal function and the diurnal variations in the maternal reproductive hormones, eight pregnant females (ORPRC) were bled (2.5 ml) at 0730 and 1530 h from Days 45 to 60, and dexamethasone (Azium, Schering Corp., Kenilworth, NJ) was injected twice daily at either of two dose levels (0.5 or 1.0 mg, i.m., four animals each) after taking each blood sample on Day 50 to 55. This procedure was repeated at the higher dose level in six of the eight animals on Days 120 to 134, and dexamethasone was injected twice daily on Days 124 to 130.

Nine ovariectomized females at the ORPRC were treated with Silastic capsules containing crystalline progesterone to test the hypothesis that the nocturnal increments in progesterone were the resultant of increased binding to serum proteins at a time when serum cortisol levels were at their lowest value. Hence single 4 cm capsules were implanted s.c., and serum samples were obtained at 0430, 1030, 1630, and 2230 h for 2 days after each implant had been in place for 48 h. Similar capsules were implanted in five rhesus monkeys in which the pituitary stalks had been transected (Norman et al., 1980), and blood samples were taken at 0800 and 2100 h to determine if nocturnal increments in progesterone were present in animals without diurnal cortisol rhythms.

Serum was stored at −20°C until assayed for E_2 (Korenman et al., 1974), progesterone (Surve et al., 1976), cortisol (Krey et al., 1975), chorionic gonadotropin (rhCG) (Atkinson et al., 1975; Niswender et al., 1971), and prolactin (Quadri and Spies, 1976). In selected samples progesterone and E_2 were quantified after Sephadex LH-20 column chromatography; the correlation coefficients relative to samples not purified chromatographically for E_2 and progesterone were 0.92 and 0.96, respectively. Intraassay and interassay coefficients of variation for the steroid assays did not exceed 7% and 11%. The samples selected for rhCG and prolactin quantification were analyzed in duplicate in a single assay at two different dose levels (the rhCG standard was a luteinizing hormone preparation [LER 1909-2] and the prolactin standard was HPRL-VLS4). The intraassay coefficient of variation did not exceed 10%.

Differences in the serum levels of the various hormones at different times of the day (a.m. vs p.m.) were compared with the Student's paired t test. Samples taken more frequently and those obtained before, during, and after dexamethasone treatment were compared by a one-way analysis of variance (ANOVA) with repeated measures in which time of day or treatment were the main effects tested. A Newman-Keul's test was used to determine which times differed when the variance ratio (F) was significant ($P<0.05$).

We wished to determine whether a correlation existed between the onset of diurnal E_2 release and adrenal development; hence we determined the time course of organogenesis and further adrenal development by microscopic examination of thin sections taken from representative rhesus embryos between Days 26 and 100 of development. These embryos are a part of the perinatal biology collection at the CPRC.

RESULTS

Onset of Diurnal Secretion

As shown in Fig. 1, diurnal alterations in the concentration of serum cortisol (31.6 ± 0.8 μg/dl, a.m., vs 22.9 ± 0.7 μg/dl, p.m.; grand mean ± SEM) in six monkeys were evident during the first trimester of pregnancy. In contrast, an apparent inverse rhythm in serum progesterone was initially demonstrable on Day 35 of gestation, when the evening level was significantly higher than the morning level (10.9 ± 1.6 ng/ml, p.m., vs 5.25 ± 1.2 ng/ml, a.m.; $t = 3.02$, $P<0.05$). This pattern continued

through Day 60 despite the gradual reduction in serum progesterone typical of this stage of gestation, and was observed thereafter through Day 150 in 10 other animals bled at infrequent intervals (data not shown). A significant morning elevation in serum E_2 was first observed on Day 37 (87 ± 10 pg/ml, a.m., vs 70 ± 6 pg/ml, p.m.; t = 5.82, P<0.01). The magnitude of the E_2 fluctuation increased with time and was superimposed on the previously described gradual increase in serum E_2 (Atkinson et al., 1975) over the next 23 days. The a.m. increment in serum E_2 was found thereafter to Day 150 (data not shown). Serum levels of rhCG declined from >1000 μg/ml to <3 μg/ml on Day 40, and no significant alterations in the levels ot prolactin were observed over this interval (15.4 ± 2.6 ng/ml, a.m., vs 19.8 ± 3.6 ng/ml, p.m.; grand mean ± SEM; data not shown).

Sequential Sampling at Close Intervals

The time courses of cortisol, progesterone, and E_2 as determined in samples obtained at 3 h intervals over a 48 h period on approximately Day 30 of gestation in eight females are demonstrated in Fig. 2a. The peak and nadir in serum cortisol concentrations were observed at 0600 and 2100 h on both days, whereas the peak

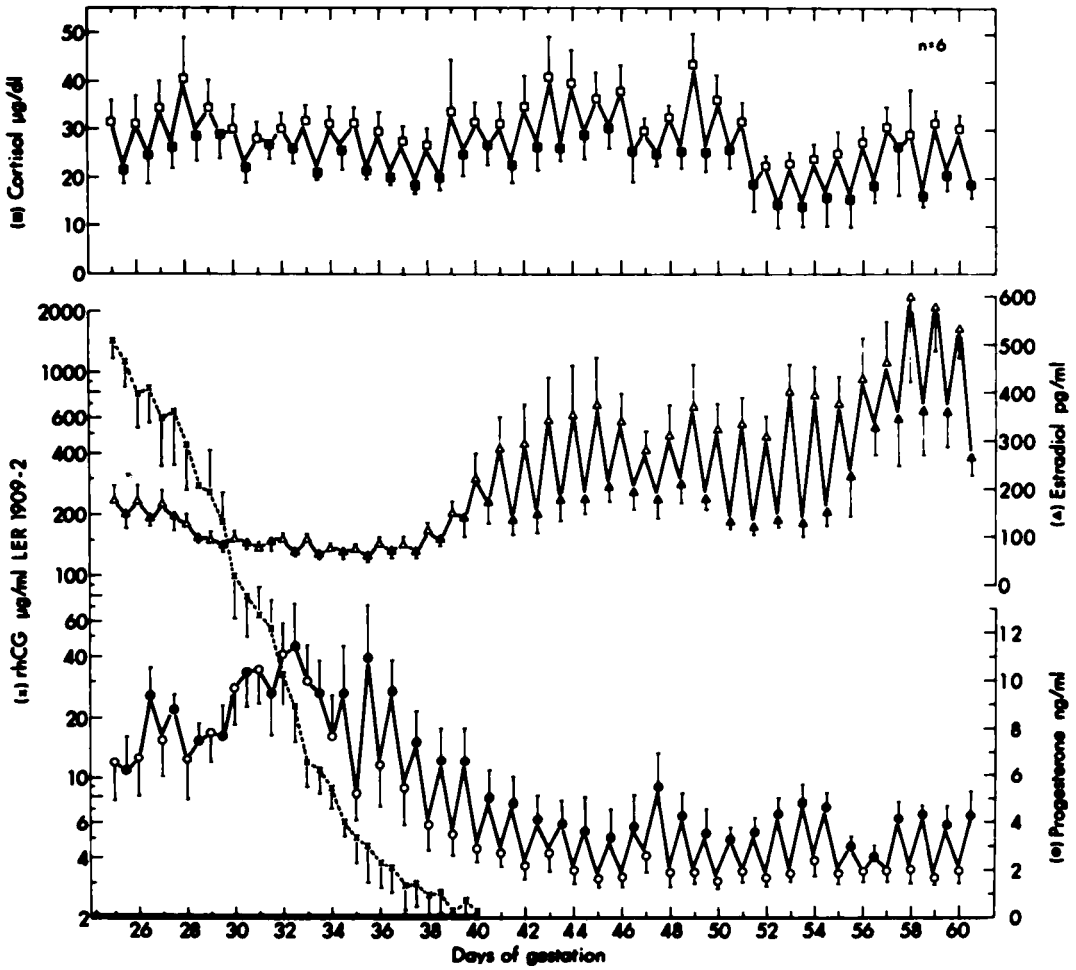

FIG. 1. Average cortisol, progesterone, estradiol, and chorionic gonadotropin (rhCG) concentrations in serum from six rhesus macaques. Serum was collected at 0730 h (open symbols) and 1530 h (closed symbols) each day from Day 25 to Day 60 of gestation. The dark bar at the bottom indicates the limit of gonadotropin detection, and the vertical lines above and below the symbols reflect the SEM.

(15.41 ± 2.1 ng/ml) and nadir (10.10 ± 1.42 ng/ml) in progesterone were opposite in phase, i.e., occurred at 2100 and 0600 h. No diurnal fluctuations in serum E_2 were observed (Fig. 2a), nor was such a pattern indicated over time in the levels of either circulating rhCG or prolactin (data not shown).

Subsequent blood samples were obtained in an identical fashion from Day 47 to Day 50 and from Day 110 to Day 116 of gestation. When the data from each bleeding sequence were compared by ANOVA, the groups were not different, and so the data were pooled for analysis of time effects (Fig. 2b). The inverse relationship between cortisol and progesterone observed on Day 30 was still evident, although the concentrations of progesterone were only ∿50% of those noted earlier in gestation. In contrast to the lack of an earlier E_2 rhythm, dramatic alterations in serum E_2 occurred; the maximum and minimum concentrations coincided with those of serum cortisol. Serum prolactin concentrations again indicated no consistent time-related alterations at either stage of gestation, and rhCG was undetectable after 40 days.

Dexamethasone Treatment

Cortisol secretion was reduced to 30% of pretreatment levels in the morning and to almost undetectable levels in the evening in the eight females given either 0.5 or 1.0 mg of dexamethasone twice daily on Days 50 to 55 of gestation (data not shown). Such treatment had no effect on the diurnal secretion of either progesterone or estrogen, although the peak and nadir in serum E_2 were reduced by 30% and 50%, respectively. In contrast, cortisol secretion was markedly reduced in the six females treated with 1.0 mg of dexamethasone on Days 124 to 130 (Fig. 3). Cessation of adrenal activity was accompanied by a parallel fall in serum E_2 levels from about 700 pg/ml to less than 200 pg/ml, and there was no indication of diurnal variation. A comparison of the concentrations of progesterone levels before, during, and after treatment by ANOVA and Newman-Keul's test at each point indicated that the inverse progesterone pattern was not abolished until Day 128, whereas the cortisol diurnal pattern was disrupted within 24 h, although complete suppression was not achieved until the evening of Day 126. Reinitiation of the diurnal release of cortisol, evident within 48

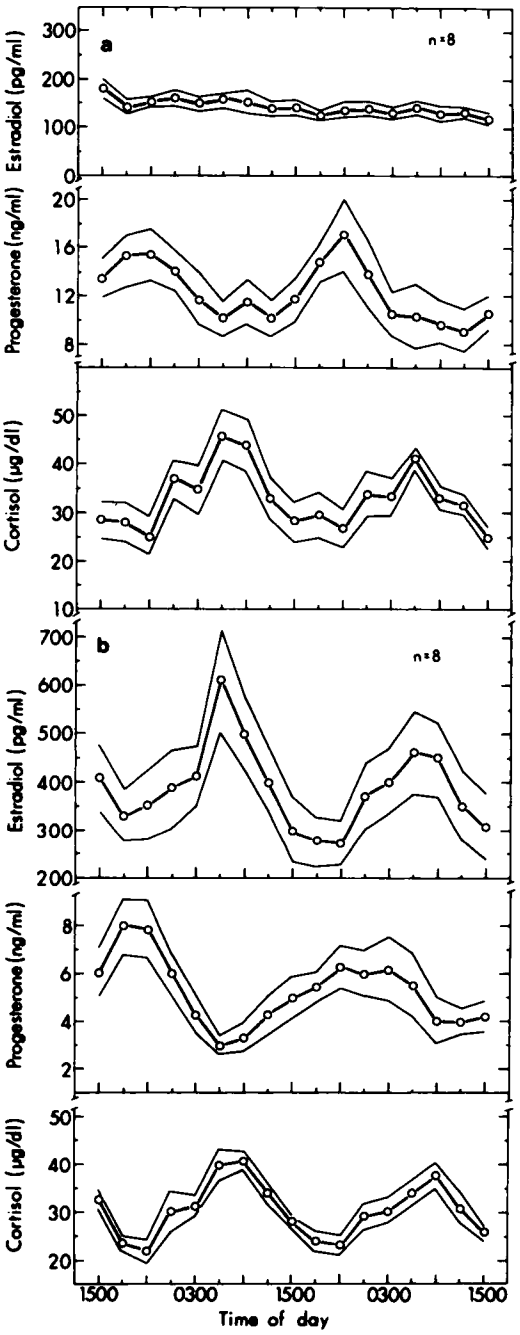

FIG. 2. a) Average cortisol, progesterone, and estradiol concentrations in serum collected from eight rhesus macaques at 3 h intervals over 48 h on Days 28 to 31 of gestation. b) Average cortisol, progesterone, and estradiol concentrations in serum collected at 3 h intervals over 48 h on Days 47 to 51 (n = 4) and Days 107 to 116 (n = 4) of gestation. Data were combined, and the SEM is indicated as for Fig. 1.

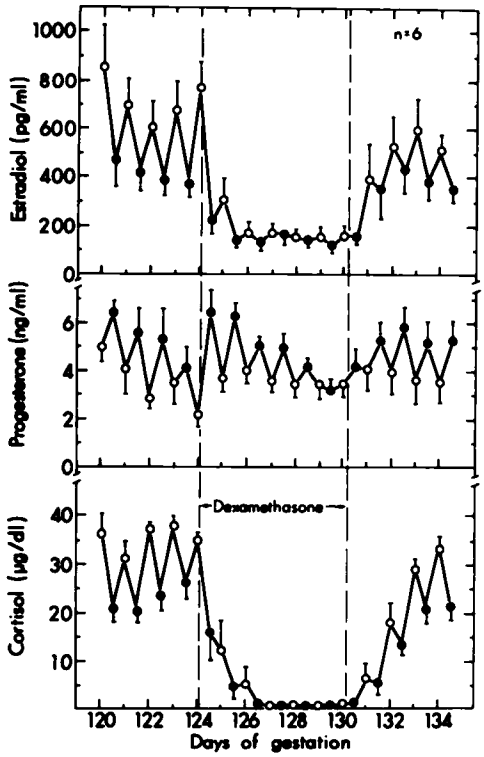

FIG. 3. Average cortisol, progesterone, and estradiol concentrations in serum obtained at 0730 h (○) and 1530 h (●) beginning on Day 120 of gestation. Dexamethasone (1.0 mg, twice daily) was injected i.m. into each animal from Day 124 to Day 130. The vertical lines above and below each symbol reflect the SEM.

h after the last dexamethasone treatment, was accompanied by the return of the pretreatment oscillations in serum E_2 and progesterone on Day 131, although E_2 levels were slightly reduced.

Higher doses of dexamethasone were not administered to avoid possible fetal death, and all animals delivered normal offspring.

Progesterone Treatment of Ovariectomized Animals

Since the preceding procedures yielded results suggesting an interplay between the serum concentrations of cortisol and progesterone, the question was approached more directly in nine ovariectomized animals given Silastic implants of progesterone. These implants were intended to act as a steady source of progesterone while normal diurnal adrenal activity continued unabated. As shown in Fig. 4, such treatment resulted in a diurnal pattern of serum progesterone that was inversely related to that of cortisol. Although the difference between the maximum and minimum concentrations of progesterone was smaller than that observed in pregnancy, it was significant ($F = 4.49$, $P<0.01$) and the timing of the peak and nadir values of the two steroids corresponded well with those observed during gestation (cf Fig. 2) when the difference in sampling frequency is considered. Furthermore, serum levels of progesterone were relatively constant and no diurnal variation was evident over an 8 day sampling period (6.82 ± 0.25 ng/ml, a.m., vs 6.73 ± 0.22 ng/ml, p.m.; data not shown) in five ovariectomized animals subjected to pituitary stalk section and treated with identical Silastic implants.

Morphological Observations

No adrenal tissue could be identified in the 28-day-old embryo, although the adrenal anlagen were present in 29–32-day-old embryos. A definitive capsule formed around the clearly differentiated gland by Days 35 to 39 (Fig. 5a), and the gland had increased in size without discernible zonation. During the next 7 to 10 days, the fetal zone underwent extensive cellular and nuclear hypertrophy, as well as hyperplasia (Fig. 5b). At an equivalent magnifi-

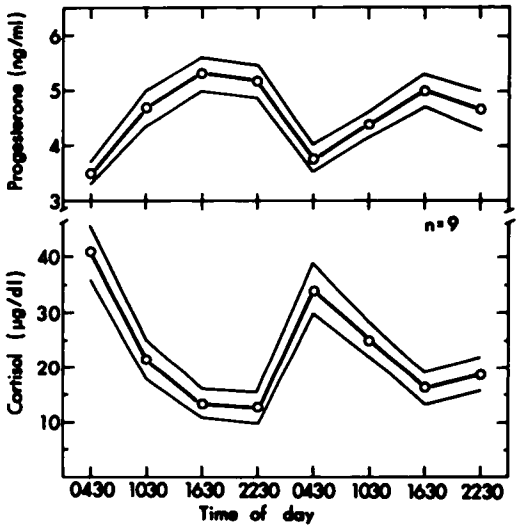

FIG. 4. Average cortisol and progesterone levels in serum from nine ovariectomized monkeys, each treated with one 4 cm long Silastic capsule containing progesterone. Samples were taken at 6 h intervals over 48 h, after the implant had been in place for 48 h. The SEM is indicated as noted for Fig. 2.

cation of 40x, the adrenal in the 42–46-day-old embryo filled most of the field and represented about one-eighth of the total gland in this cross section, whereas the entire gland was encompassed at the earlier time. In addition, a definitive cortex, consisting of only a few cell layers immediately underlying the capsule, was demonstrable in some sections at the later gestational age. The morphological characteristics of the gland, other than continued growth of the fetal zone with much smaller increments in the definitive zone, did not change significantly through Day 100.

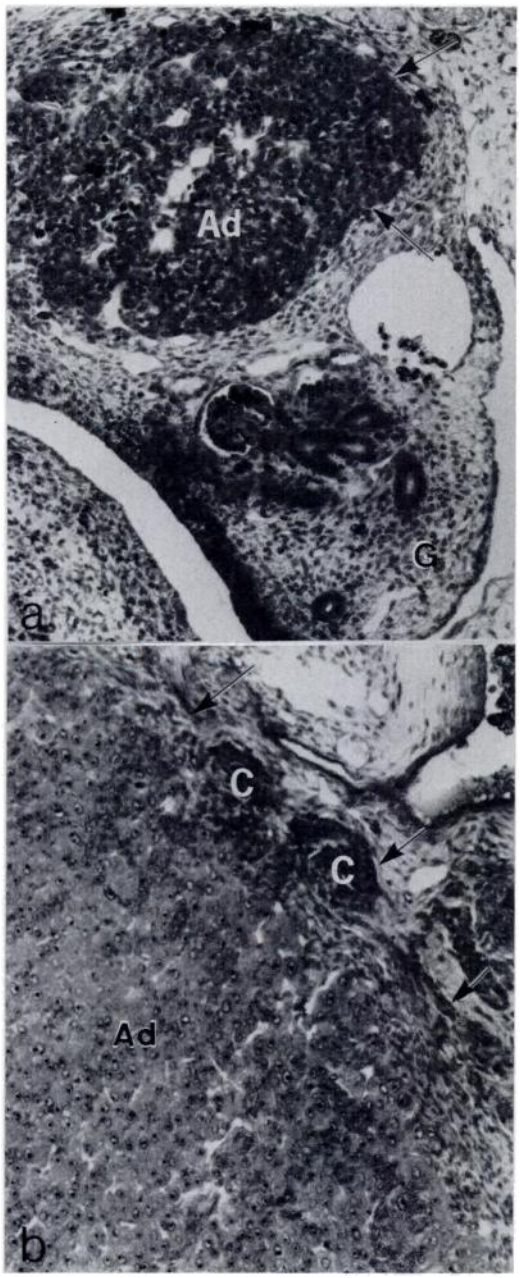

FIG 5. Cross sections through the adrenals of a 35–39-day-old embryo (a) and 42–46-day-old embryo (b). Ad, adrenal gland; G, undifferentiated gonad; Ch, chromaffin cells. Arrows indicate the limiting capsule. X38.

DISCUSSION

These studies have demonstrated that coincident diurnal variations in the maternal serum concentrations of E_2 and cortisol (a.m. maxima) begin on about Day 40 of gestation, whereas serum progesterone levels show an inverse rhythm (p.m. maxima) with respect to cortisol which is present by at least Day 30. The relationships of the diurnal patterns in the three hormones remain consistent throughout gestation. This finding confirms and extends, in a larger series of animals, the original description of these rhythms in the immediate preparturient period in four animals (Sholl et al., 1979a,b) and in 11 paired samples obtained from chronically chaired animals on Days 120 to 130 of gestation (Challis et al., 1980).

The absence of diurnal changes in maternal serum E_2 prior to Day 40 suggests that the onset of this pattern requires a functional fetal adrenal since normal maternal adrenal function continues unabated from Day 25 to Day 150. The dramatic fall in maternal E_2 observed by Atkinson et al. (1975) after ovariectomy on Day 23 is further evidence that the estrogen pattern depends on the maternal ovaries rather than the maternal adrenal or fetoplacental unit during early gestation. Histological examination of rhesus embryos has indicated that adrenal differentiation occurs during the 35 to 40 day gestational period and that organogenesis was followed by rapid and dramatic growth of the fetal zone between Days 40 and 50. While admittedly indirect, these observations lend credence to the hypothesis of fetal adrenal activation. In chronically catheterized monkeys, Séron-Ferré et al. (1978) demonstrated a diurnal rhythm in fetal cortisol on about Day 150 which was similar to that of the mother, but Challis et al. (1980) could not find a similar variation in animals earlier in gestation; they have suggested that fetal adrenal function may be higher in the p.m. than a.m., on the basis of the hypothesis that the fetal pituitary-adrenal axis is relieved of maternal cortisol suppression

in the p.m. Since dexamethasone treatment in the present study suppressed both maternal and fetal adrenal (Challis et al., 1974), the loss of the coincident E_2 and cortisol variations seen here cannot be attributed to either alone. Alternatively, the onset of diurnal E_2 levels may follow the maturation of the placental aromatization complex and subsequent metabolism of estrogen precursors from the maternal adrenal, although the rapid fall in E_2 levels after fetectomy later in gestation argues for a substantial fetal adrenal contribution from Day 70 onward (Tullner and Hodgen, 1974).

The serum concentrations of progesterone show a diurnal pattern that is the inverse of the E_2 and cortisol pattern, and this inverse relationship is demonstrable over a wide range of progesterone concentrations, e.g., 10 to 16 ng/ml on Day 30 and 1 to 6 ng/ml during the remainder of gestation. Sholl et al. (1979a) have suggested that the diurnal alterations in maternal serum progesterone in late gestation may reflect a variation in placental progesterone production, either as a result of altered blood flow or through changes in placental steroidogenesis induced by prolactin or other protein hormones. Although our study indicated that there are no diurnal alterations in the serum levels of either prolactin or rhCG during those periods studied in adequate detail (30, 50, and 110 days), it is not possible to rule out a possible contribution of diurnal alterations in uterine blood flow (Harbert and Zuspan, 1977). Alternatively, Challis et al. (1980), on the basis of a correlation between dehydroepiandrosterone sulfate and progesterone increments in evening fetal samples, have suggested that these distinctive hormone profiles were the consequence of altered fetal adrenal progesterone release. However, these suggestions cannot be the complete explanation since nocturnal increments in progesterone have been observed in other conditions when serum progesterone levels have been elevated in the absence of the fetal-placental unit. A similar pattern has been noted in intact animals during the luteal phase, and the pattern is reversed after ovariectomy, although the amounts released by the adrenal in the morning are very small (Spies et al., 1974). In our study ovariectomized females treated with progesterone implants also had a diurnal progesterone pattern, whereas ovariectomized females so treated after pituitary stalk section to remove diurnal adrenal function (Norman et al., 1980) showed no such pattern. The presence of similar diurnal relationships between cortisol and progesterone in such divergent models supports the hypothesis that the two contrasting patterns are the result of direct cortisol-progesterone-protein interactions.

We have postulated that the normal diurnal secretion of cortisol by the maternal adrenal alters the displacement of progesterone from common binding sites on serum proteins, particularly cortisol-binding globulin (CBG), and thus the concentration of circulating progesterone varies as the metabolic clearance of the steroid changes after alterations in binding. We expected that suppression of both the fetal and maternal adrenals with dexamethasone (Challis et al., 1974; Walsh et al., 1979) would result in the rapid and unambiguous loss of the nocturnal progesterone increment. This increment did eventually disappear, but we have no unassailable explanation for either the continued rhythm seen relative to the immediate disruption in cortisol release or the absence of elevated basal progesterone levels as cortisol disappeared from the circulation. The progesterone pattern promptly reappeared with the onset of adrenal activity after treatment. While not conclusive, these observations support earlier in vitro evidence that increased levels of cortisol decrease progesterone binding to CBG (DeMoor and Steeno, 1965; Westphal, 1967) and agree with in vivo evidence (Albrecht et al., 1978; Pepe et al., 1977) indicating that variations induced in serum cortisol levels by stress or drug treatment in baboons are accompanied by concurrent and inverse changes in the clearance rate and levels of circulating progesterone.

Although we have verified the existence of diurnal rhythms in the serum concentrations of cortisol, E_2, and progesterone during much of gestation, we do not yet know their physiological importance. Challis et al. (1980) have suggested that maternal adrenal function may alter the secretory activity of the fetal pituitary-adrenal axis; the role, if any, that such changes play in fetal growth and development is unknown. It is known that primates, especially rhesus macaques, have a higher incidence of births in the early morning hours (colony records of the ORPRC; King, 1956), but we do not have sufficient data to determine whether the nocturnal elevation in progesterone has any relationship to the duration of gestation, onset of parturition, or other features of normal pregnancy.

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