On Circulating Sex Steroids During the Estrous Cycle and the Early Pseudopregnancy in the Rat with Special Reference to its Luteal Activation*

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Synopsis

Estradiol-17 β (E-2), estrone(E-1), progesterone(P), and 20 α -hydroxypregn-4-en-3-one(20 α -OHP) were measured in peripheral plasma on every 2–4 hr throughout the 4-day estrous cycle and the early pseudopregnancy in rats illuminated 14 hr a day (0500 to 1900). The radioimmunoassay and the competitive protein binding assay were respectively used for the measurements of estrogens and progestins.

In the estrous cycle, E-2 showed a single rise spread from 1600 of diestrus to 1500 of proestrus, where a maximum value at around 0800 of proestrus was followed by an abrupt drop to the basal level by 2000 of the same day. Increasing tendencies of E-1 level at around 2000 of diestrus and around 1800 of proestrus were found, though showing relatively large variations. In progestins in the cycle, P showed significant rises in the evening between 1600 and 2200 every other day, i.e., on the days of proestrus and metestrus. Since daily rises of 20 α -OHP were observed between 1600 and 2200 except on the day of proestrus, the total progestin (P + 20 α -OHP) manifested clear daily rhythmic fluctuation, having a peak every evening in the cycle.

In the first 3 days of pseudopregnancy(PSP), E-2 remained essentially unchanged at a low level, but E-1 showed a significant rise from the late afternoon to the night of Day 1 of PSP. On the other hand, P showed significant rises in the morning (0800) and a much higher rise in the evening (2000) on Day 1 of PSP. The latter rise was also accompanied with the increasing tendency of 20 α -OHP. The total progestin did not show apparent daily rhythm during the early PSP. It is revealed that sex steroids patterns during the early PSP are not so simple as hertofore assumed.

Induction of PSP by the single injection of P was possible only at the fixed time (0700–1000) on the day of estrus throughout the estrous cycle. An important role of extra-periodical P secretion found on the day following the cervical stimulation (Day 1 of PSP) was suggested as a possible intervening mechanism for inducing PSP.

To better understand complex aspects of the relationship between hypothalamo-hypophyseal hormones concerning the gonadal axis and ovarian hormones, it is urgent to make clear

the precise change in the circulating level of sex steroids as the feedback signals.

In the past few years, the revolutionary developments of assay methods of steroid hormones have been achieved. The so called "competitive radioassays," owing to the high sensitivity and rapidity of procedures, now make it possible to determine the patterns of the circulating estrogen or progestin level in various reproductive stages even in the small laboratory animals.

Applying these assay methods, in this paper

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the authors determine peripheral plasma estrogens (estrone and estradiol-17 β) and progestins (progesterone and 20 α -hydroxypregn-4-en-3-one) simultaneously at short enough intervals to reveal their precise patterns during the estrous cycle and the early stage of pseudopregnancy in the rat. In addition, a possible role of progesterone, secreted extra-periodically on the day following the cervical stimulation, was discussed in relation to the induction of pseudopregnancy, that is, the luteal activation in the estrous cycle of the rat.

Materials and Methods

Animals

Virgin female Wistar rats bred in our laboratory (200–300 g), were housed in an air-conditioned room

at $25 \pm 2^\circ\text{C}$, under a controlled lighting condition of 14 hr light (0500–1900) and 10 hr darkness. A rat chow (CA-1, CLEA Co., Tokyo) and water were given *ad libitum*. Vaginal smears were taken every morning, and only those rats which had shown at least three consecutive regular 4-day cycles were used in the present study.

Pseudopregnancy was induced by the mechanical stimulation of tapping the uterine cervix with a glass rod more than 200 times at 1730 on the day of vaginal proestrus. That almost all the animals thus treated result in the pseudopregnancy, has previously been confirmed in our laboratory. The day of stimulation was designated as day 0 of pseudopregnancy.

Collection of systemic blood

Systemic blood was taken from the inferior cava under ether anesthesia at various times as shown in Table 1. Immediately after collection, the blood was centrifuged at 3,000 rpm for 15 min. Plasma samples were divided into two portions; one was used for estrogen assay, and the other for assay of progestins. Plasma samples were stored at -20°C

Table 1. Blood sampling times in the 4-day cycling rats and during the early pseudopregnancy.

Day of Cycle	Time of Blood Collection	No. of Rats	Day of PSP*	Time of Blood Collection	No. of Rats
Proestrus (Day P)	0200	4	0 (Proestrus)	1830	3
	0500	4		2000	3
	0800	4		2200	3
	1100	3	1		
	1400	3		0200	3
	1600	3		0600	3
	1800	3		0900	3
	2000	3		1200	3
	2200	3		1600	3
Estrus (Day E)				2000	3
	0200	3	2		
	0600	2		930	3
	1200	3		1200	1
	1600	3		1600	3
Metestrus (Day M)	2000	3	3	2000	3
		2		0830	3
	0400	2		1200	3
	0800	2			
	1200	3	PSP*; Pseudopregnancy		
Diestrus (Day D)	1600	3			
	2000	4			
		2			
	0400	3			
	0800				
	1200	3			
	1600	3			
	2000	3			

until processed.

Extraction and fractionation of steroids from plasma

Rat peripheral plasma samples (1 ml each) were added to the estrogen extraction tubes containing of estrone-6,7-³H (NEN, SA 40Ci/mM) and estradiol-17 β -6, 7-³H (NEN, SA 40 Ci/mM), 500 cpm each and added to the progestin extraction tubes containing of progesterone-1, 2-³H (NEN, SA 50.3 Ci/mM) and 20 α -hydroxypregn-4-en-3-one-1, 2-³H [prepared from progesterone-³H according to a method described by Wiest (1969)], 500 cpm each.

Steroids were three times extracted with four fold volume of diethyl ether-benzene (1:1). Combined extracts following evaporation to dryness, were subjected to column chromatography (Sephadex LH-20) using benzene-methanol (85:15) as an elution solvent for separation of estrone, estradiol-17 β and neutral steroids fraction. The neutral fraction containing progestins was subjected to additional Sephadex LH-20 column chromatography (Hexene: Methanol: Benzene = 80:10:10) for the separation of progesterone and 20 α -hydroxypregn-4-en-3-one (20 α -OHP).

Steroid Assays

Estrogen

Estrone (E-1) and estradiol-17 β (E-2) were measured by radioimmunoassay (RIA) using an antiserum to E-2 which cross reacts with E-1. The antiserum to E-2 was kindly supplied by Dr. B. V. Caldwell mediated by Drs. G. Mikhail and W. G. Wiest. The dilution of the antiserum used was 1:55,000 for both estrogens. The method of RIA used was that developed by Hotchkiss *et al.* (1971) with minor modifications.

Progestins

Progesterone was measured by competitive protein binding assay as previously described by Horikoshi and Wiest (1971). In this experiment, one modification was introduced; the serum of female rats, adrenalectomized, one week before was used for steroid binding protein instead of using human female plasma. Fractions of 20 α -OHP were oxidized with chromic acid and measured as progesterone, according to the method as previously described (Horikoshi and Wiest, 1971). The workable range of the assay was from 1 to 35 ng progesterone.

Measurements of radioactivity

Counting and data processing were carried out as previously reported (Horikoshi and Wiest, 1971). Tritium counting efficiency in the Packard scintillation

spectrometer Model 2311 was 25% for all samples.

Progesterone administration

To test prolongation of the diestrous period, progesterone was injected subcutaneously only once (10 mg/0.2 ml in sesame oil) at various times during the estrous cycle.

Results and Discussion

Changes in circulating levels of plasma estrogens and progestins in the 4-day cycling rat are given in Figures 1 and 2, where the concentrations of E-1, E-2 and the total estrogen (E-1 + E-2) expressed in pg/ml plasma, and the concentrations of progesterone, 20 α -OHP and the total progestin (progesterone + 20 α -OHP) expressed in ng/ml plasma are separately plotted against chronological time when the blood is collected during the four days characterized by the vaginal cytology. The so called "critical period" for the ovulatory LH surge and the time of the ovulation have previously been confirmed respectively as 1400–1600 of Day P and 0000–0300 of Day E in our rats under the photoregimen of 14L/10D with light on at 0500 (Eto *et al.*, 1958).

Similarly, changes in circulating levels of the sex steroids during the first 4 days of pseudopregnancy including the day of cervical stimulation, are given in Figures 3 and 4, where the mean levels of respective steroids on the corresponding days in the estrous cycle are also shown in broken lines for the comparison between them.

Prolongation of the diestrous period by a single administration of progesterone is summarized in Figure 5, where the mean durations of the diestrous smear after the injection are plotted against the time of injection during the estrous cycle, and each point is compiled at least from 3 experiments.

In the rat, an increase of estrogen secretion during the follicular phase has been either predicted by the indirect parameters (Astwood, 1939; Schwartz, 1964; Barnea *et al.*, 1968), or

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5. Effects of a single administration of progesterone on prolongation of diestrus period at varying times in the 4-day cycling rats.

assessed directly by the estrogen assays in the ovarian venous blood (Hori *et al.*, 1968; Yoshinaga *et al.*, 1969; Shaikh, 1971) or in the peripheral blood (Brown-Grant *et al.*, 1970), though these data have been mainly compiled only in the stages of diestrus and proestrus. As seen in Figure 1-a, our data clearly indicate that the circulating E-2 shows only one rise spreading from 1600 of Day D to 1400 of Day P throughout the estrous cycle. This picture is good accord not only with the change of uterine weight including the luminal fluid, but also with the secretory pattern of estrogen reported by the previous workers. Since the ovulatory surge of LH release has been proved to occur from 1600 to 2000 of Day P (Monroe *et al.*, 1968; Goldman *et al.*, 1969; Gay *et al.*, 1970; Brown-Grant *et al.*, 1970; Barraclough *et al.*, 1971), the decline of the increased secretion of E-2 as indicated by the abrupt drop in E-2

level, seems to start before the LH surge. Our data are compatible with a hypothesis that estrogen acts as a trigger for the ovulatory release of LH (Everett, 1948; Miyake, 1968; Kobayashi *et al.*, 1969).

The circulating E-1, another component of estrogens having a far less potent estrogenicity, has not been separately measured until recent years. Recently, using RIA Shaikh (1971) has reported the change of the E-1 level in the ovarian venous blood of the rat, where it shows a very similar pattern to that of E-2. The blood samplings of him, however, are not frequent enough to clarify the details of E-1 change throughout the estrous cycle. Baranczuk and Greenwald (1973) have reported a daily nocturnal rise of E-1 having a peak at around 2200 every day in the cycling hamster. As shown in Figure 1-b, the change of circulating E-1 level in the cycling rat is slightly but distinctly different from that of

Fig. 1-4. Changes in circulating plasma estrogens and progestins concentrations (means with standard errors) in the 4-day cycling rats (Fig. 1. and Fig. 2.) and during the first 4 days of pseudopregnancy (Fig. 3. and Fig. 4.). The total estrogens and progestins were calculated for each sample as estrone + estradiol-17 β and progesterone + 20 α -hydroxypregn-4-en-3-one, respectively.

E-2 and showing two separated peaks with a short intermittence. Furthermore those, daily rhythmic fluctuations which are found either in the E-1 level of the hamster (Baranczuk and Greenwald, 1973) or in the total progestins of the rat in the present study, are not likely to exist not only in the E-1 level but also in the level of the total estrogen as shown in Figure 1-c.

In the circulating progestins as shown in Figure 2, progesterone markedly increases twice in the estrous cycle, on the late evenings of the metestrus and proestrus, as have been revealed in the surveys in the ovarian venous blood (Eto *et al.*, 1962; Telegdy and Endroczi, 1963; Hashimoto *et al.*, 1968; Uchida *et al.*, 1969; Goldman *et al.*, 1969; Piacsek *et al.*, 1971; Barraclough *et al.*, 1971). In the present study, another peak, a small but statistically significant rise in progesterone, is indicated on the morning of Day P in addition to the two large peaks above mentioned. Although a main large peak of progesterone in the evening of Day P has been accepted as a consequence of the action of LH released at the ovulatory surge, the small peak in the morning would be suggestive of the facilitatory influence on the regulatory mechanism of ovulating LH surge as well as the action of the preovulatory estrogen. Because progesterone administered on Day P has been proved to advance not only the ovulation by one day in 5-day cycling rats (Everett, 1948), but also the time of LH release for few hours in 4-day cycling rats (Zeilmaker, 1966; Kobayashi *et al.*, 1970).

It is noteworthy that the circulating total progestin, including biologically inactive progestin, i.e., 20α -OHP, clearly manifests daily rhythmic rise between 1600 and 2000 every day in the cycle. Of these 4 rises in the cycle, the peaks on Day E and Day D are mostly occupied by 20α -OHP alone, that on Day P by progesterone alone and that on Day M by both progestins.

In the cycling rats under a fixed photoregimen (14L/10D), Everett and Sawyer (1950)

have firstly indicated the existence of a potential 24 hr periodicity in LH releasing mechanism by investigating the blockade of ovulation with pentobarbital anesthesia on the day of proestrus. Recently, applying the anesthesia at different times of day throughout the estrous cycle, Okamoto *et al.*, (1972) have clarified that the ovulation is delayed by one day when the anesthesia is given at a limited time band (1200–1600) of any day in the cycle except the day of estrus. In this connection, Kawakami *et al.* (1970) have found an increase of polynuclear activity in the arcuate nucleus of the hypothalamus from 1300 to 1700 every afternoon in the cycling rat.

These findings are suggestive of the existence of periodical excitation in CNS which may correlate with some daily rhythmic change for hypothalamo-hypophysial activity concerning gonadotrophin secretions during the cycle. As supporting evidence, Ramirez and Sawyer (1965) have found a daily rhythmic change in the content of hypothalamic LH-RF, and Gay *et al.* (1970) have found a daily diurnal rise of plasma LH in the cycle, though its amplitude is very small except that on Day P. Therefore, a daily rhythm of the total progestin presented in this paper, may well be a peripheral reflexion of the endogenous rhythm of neuroendocrine regulating mechanism on the gonadal axis in the cycling rat.

The levels of sex steroids during pseudopregnancy (PSP) and pregnancy in the rat have been measured mainly in the ovarian venous blood by many investigators (Eto *et al.*, 1962; Fajer and Barraclough, 1967; Hashimoto *et al.*, 1968; Yoshinaga *et al.*, 1969; Uchida *et al.*, 1970; Shaikh, 1971; Bartosik and Szarowski, 1973). In these studies, however, little attention has been paid on the detailed secretory patterns at the early stage of PSP or pregnancy. In the present study our attention is mainly directed to the changes of circulating sex steroids during the very early stage of PSP to reveal the relationship between the sex steroids and the activation of luteal function. As shown in Figures 3 and 4, remarkable

effects of the cervical stimulation, are found in the plasma progesterone, 20α -OHP and E-1 levels as early as Day 1 of PSP. Especially, two peaks of progesterone on Day 1 of PSP are quite different from the secretory pattern found on the corresponding day in the cycle (Day of estrus).

Progesterone secretion has been thought to be low in the early PSP or the early pregnancy (Uchida *et al.*, 1970; Bartosik and Szarowski, 1973), because the growth of the functional corpora lutea closely relates to the increase in secretion of progesterone (Uchida *et al.*, 1970). The reason why the previous workers have overlooked the significant increase of progesterone secretion in Day 1 of PSP, may be attributable firstly to the fact that the determinations of their studies were not frequent enough to reveal delicate changes, and secondly to the fact that progesterone in the early PSP does not increase in correspondence with the time of increase during the estrous cycle.

Thus, it is strongly suggested that the daily rhythm of the total progestin level in the cycle becomes undetected during the early stage of the PSP at least. Although plasma E-1 also shows a significant rise on Day 1 of PSP, its physiologic significance can not be predicted at present.

From the whole of our present data, it may reasonably be concluded that the secretory patterns of sex steroids, especially on progesterone, during the early stage of PSP are not so simple as have previously been assumed.

Since the plasma progesterone revealed a peculiar change on Day 1 of PSP, we tested the prolongation of the diestrous period by administering a single dose of progesterone at different times throughout the cycle in order to examine the possible physiological role of progesterone on the activation of the luteal function. As seen in Figure 5, a PSP-like continuous diestrus was obtained in high incidence (50–100%) by the injection only at the narrow time band between 0700 and 1000 of Day E. This time is just coincide with the

first peak of progesterone rises found on the corresponding day in the PSP (Day 1 of PSP). Furthermore, the rats of this PSP-like diestrus were able to induce the deciduomata by the traumatization of their uteri 4 days after the injection, and the duration of PSP-like diestrous periods was not different from that of the PSP in the rat of our colony. Therefore, it may be admitted that these rats have the continual luteal function similar to the normal PSP. Rothchild and Schubert (1962) have incidentally reported the induction of PSP by the single injection of progesterone in the morning of the estrous rats having 4, 5 or 6-day cycle, although the effective time course has not been investigated in detail. The mechanism of the injected progesterone to cause PSP in our case may not be a direct one to the ovary, but it may be the activation of continued secretion of progesterone of the corpus luteum by luteotrophin, a mechanism involving the effect on the central neuro-endocrine system.

From these findings, we are tempted to speculate a possible role of progesterone secreted on the day following the cervical stimulation as an indispensable entity for establishing the state of the PSP, i.e., the activation of the continued luteal function in the rat. That is, the first rise of progesterone on the early morning next to the day of cervical stimulation (Day 1 of PSP) may exert, as is simulated by the exogenous progesterone given at the corresponding time of the estrus, the positive feedback effect on the central mechanism of luteotrophin secretion and results in the second rise of endogenous progesterone secretion on the late evening of the same day. As shown in Figure 5, the exogenous progesterone given at around 2000 of Day E fails to induce the PSP, therefore, the change in the central mechanism brought by the increased secretion of progesterone during the early morning might be a prerequisite not only for rising progesterone in the evening, but also for shifting the secretory pattern of luteotrophin, presumably

prolactin, from that of the estrous cycle (Gay *et al.*, 1970) to the peculiar daily nocturnal surging pattern of prolactin which has recently been revealed by Freeman and Neill (1972).

Our view is still lacking the conclusive supporting evidence. Moreover, as well as progesterone, E-1 level shows a significant increase on the day following the cervical stimulation (Day 1 of PSP). Further studies will be needed to test our hypothesis, however, progesterone secretion at the very early stage of PSP would be valid as an intervening mechanism for the luteal activation.

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