Sex difference in resting pituitary-adrenal function in the rat

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It is now apparent that female rats show a greater adrenal cortical secretory response to stress than do males (23, 27, 28). However, it is not clear whether a similar sex difference in adrenal cortical function exists under resting conditions. Following rapid decapitation, Sakiz (28) found higher adrenal concentrations of corticosterone in females than in males, with no difference in plasma levels, and Kitay (23) reported higher levels of plasma corticosterone in females than in males. In rats anesthetized with pentobarbital, no sex difference in concentration of plasma corticosterone was reported during the morning hours by Guillemin et al. (13) or during several intervals of a 24-hr period by McCarthy and co-workers (25). According to Halberg et al. (19), higher peaks in resting levels of adrenal corticosterone are found in female than in male mice.

In preliminary experiments in this laboratory, female rats generally had higher levels of plasma corticosterone than did males following rapid decapitation. The present experiments were designed to explore further the possibility of a sex difference in pituitary-adrenal function under resting conditions. Because of an approximate 24-hr, or circadian (15), rhythm of adrenal cortical secretion in the rat (14), comparisons between males and females were made at 2-hr intervals throughout controlled 24-hr light-dark cycles. In view of the apparent absence of a sex difference in anesthetized rats (25), adrenal cortical function was also studied following the administration of pentobarbital during selected periods of the 24-hr day-night cycle. Because environmental lighting apparently exerts a synchronizing influence on the rhythm of adrenal cortical function in mice (16), several groups of rats were studied following a phase shift in lighting regimen. These studies were summarized in part in a previous report (15).

MATERIALS AND METHODS

A total of 280 adult and prepubertal Holtzman rats of both sexes were used to study the 24-hr variations in resting levels of plasma and adrenal corticosterone, pituitary concentration of adrenocorticotropic (ACTH), and circulating leukocytes. All animals were allowed a minimum of 3 weeks to adjust to laboratory conditions of controlled light and temperature. The lighting schedule consisted of 14 hr of artificial light per day, from 0400 to 1800, alternating with 10 hr of darkness. The
rats were placed in individual cages 3 days before each experiment and were given Purina chow and water ad libitum.

In the course of three experiments, intact, castrated, and sham-castrated adult and prepubertal rats of both sexes were studied. Five rats of each of these groups were killed by rapid decapitation at 4-hr intervals throughout either a 24- or 32-hr period. Adult males ranged in weight from 185 to 294 g, while females weighed from 168 to 262 g. Castration and sham-castration (incisions and manipulation of gonads without removal) were performed 20-21 days prior to autopsy. Prepubertal animals were placed in the animal quarters at 8 days of age, weaned at 18 days, and killed at 30 days. Vaginal smears were obtained from adult females for at least 3 days before each experiment and following decapitation; the remaining rats were handled daily to simulate the vaginal smear procedure.

At time of autopsy, the rats were removed individually from the animal quarters and decapitated within 20 sec. Blood was collected in heparinized beakers, a sample was taken for hematological study, and the remainder was centrifuged. Leukocyte counts were done with routine laboratory methods. Individual plasma samples were frozen and stored for determination of corticosterone in 0.5-ml aliquots according to the method of Silber et al. (31) as modified by Guillemin and co-workers (12). The results are expressed as μg corticosterone/100 ml plasma.

Immediately following decapitation, the adrenals were dissected, cleaned, weighed to the nearest 0.1 mg, ground in alcohol-saline, and frozen for subsequent fluorometric determination of corticosterone content (12, 31); the values are expressed as μg corticosterone/g adrenal.

Concurrent with the dissection of the adrenals, the pituitary was rapidly removed, the gland was weighed to the nearest 0.02 mg and ground in 1.5 ml of 0.01 or 0.1 N HCl in saline. The extracts were frozen and stored. Later, the extracts from each group were thawed, pooled, and assayed in vivo for ACTH content, using the measurement of plasma corticosterone in the 24-hr hypophysectomized rat (13). A bracketed type of assay was employed in which a single dose of pooled pituitary extract was assayed against high and low doses (0.75-0.25 μg) of USP reference standard ACTH. This dose range is one in which preliminary studies had shown the response to be linear. Fifteen minutes following the injection of either standards or unknowns into jugular veins of three to six 24-h hypophysectomized rats, blood was withdrawn from the abdominal aorta and plasma corticosterone was measured fluorometrically as described above. Potencies of ACTH activity in the pooled pituitary extracts from each group are expressed as mU ACTH/mg pituitary tissue. The precision of values obtained in bracketed assays on ten replicates done in this fashion was ±0.2 mU/mg.

To determine the effects of pentobarbital on the daily

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**Fig. 1.** Twenty-four-hour patterns of resting levels of plasma (A) and adrenal (B) corticosterone, pituitary ACTH (C), and circulating leukocytes (D) in male and female rats. In this and subsequent figures the vertical lines represent standard errors, and the horizontal black bars indicate periods of darkness.
elevation in resting levels of plasma and adrenal corticosterone, a dose of 4.5 mg/100 g body wt. was administered intraperitoneally to eight females at 1500 and to five females at 1900. Males were injected with 5.0 mg/100 g body wt.; 16 were anesthetized at 1500 and 10 at 1900. These doses of pentobarbital were in excess of those used by McCarthy and co-workers (25) to insure a deeper level of anesthesia at time of decapitation. Following injection, each rat was placed in an environment of 95% oxygen and 5% carbon dioxide, and body temperature was maintained at approximately 38 °C. Thirty minutes following administration of pentobarbital, the rats were decapitated and blood and adrenals were treated as described above for determination of corticosterone concentrations.

To study the effects of a shift in lighting regimen on the pattern of resting adrenal cortical function, 70 males and 105 females were exposed initially to light from 0400 to 1800. At the end of 3 weeks, 35 rats of each sex were blinded by bilateral optic enucleation and 35 females were ovariectomized. The intact, blinded, and ovariectomized rats were then subjected to a 9-hr shift in lighting, with light administered daily from 1300 to 0300. Following a 3-week exposure to the changed light schedule, the 24-hr patterns of plasma and adrenal corticosterone were determined as described previously.

All statistical probabilities were derived from analyses of variance.

RESULTS

Adult males and females. Figure 1 summarizes the results obtained in intact male and female rats. The times indicated in this and subsequent figures represent approximate mid-points of sampling periods which were less than 90 min in duration.

As shown in Fig. 1A, marked excursions were observed in the concentrations of plasma corticosterone in both sexes during the 32 hr of the experiment. Both males and females demonstrated the lowest levels during the 2300 and 0300 periods, and in each sex the first signs of a diurnal elevation in corticosteroid appeared at approximately 1100. Whereas there was no sex difference at 0300, the peak level of females at 1900 was markedly higher than that of males.

Fluctuations were observed in the levels of cortisol in the adrenals that were temporally related to those found in plasma (Fig. 1B). In plasma, there was no sex difference in corticosteroid concentrations in the early morning, and the subsequent peak was higher in females than in males.

Figure 1C summarizes the variations observed in pituitary ACTH concentrations in adult males and females. As with the corticosteroids, fluctuations in content of ACTH were observed, and there was an apparent sex difference in the pattern of these fluctuations. In males, the highest levels were found in glands of rats killed at the first 0700 sampling; the lowest concentrations were obtained between 1500 and 2300, during and immediately following periods when the highest concentrations of corticosteroids were observed. In contrast, pituitaries from female rats had the lowest concentrations of ACTH in the early morning and the highest at 1900 when corticosteroid levels were highest in both plasma and adrenals.

The leukocyte counts are presented in Fig. 1D. Both males and females showed signs of a 24-hr rhythm in
numbers of circulating white blood cells. In each sex the lowest values were obtained at 1900, in close temporal relationship to peak corticosteroid levels.

Castrated males and females. Figure 2 summarizes the results obtained in castrated males (studied for 24 hr) and ovariectomized females (studied for 32 hr).

The 24-hr variations in plasma corticosterone in castrated males (Fig. 2A) differed in several respects from those of intact males. The most conspicuous difference appeared at the first (0700) sampling period when the levels recorded were higher than those obtained at the same time of day from intact males. A subsequent peak did appear in the castrated males, but around 1500 instead of at 1500 as in intact males. The pattern in ovariectomized females lacked the marked afternoon elevation that was found in intact females, and its temporal and quantitative features were similar to those of intact males.

The variations in adrenal concentration of corticosterone in castrated males, Fig. 2B, were similar to those of intact males. However, the peak appeared at 1900 instead of at 1500. In contrast to the findings in plasma, high concentrations of corticosterone were not obtained at the first sampling period. The amplitude of changes in the adrenals of ovariectomized females was much reduced in comparison to that of intact males.

In intact males, the initial resting levels of plasma corticosterone in sham-operated rats were relatively high (Fig. 3A). The subsequent pattern of changes observed in this group included a secondary peak in corticosterone at 1900. The variations found in sham-ovariectomized females were quantitatively similar to those observed in intact females. Pituitaries from castrated males and females, like those of intact males, demonstrated a slight decrease in ACTH during the period of highest corticosteroid levels, a pattern similar to that seen in intact males.

Gonadectomized males and females showed decreases in circulating leukocytes during the 1100–2300 period (Fig. 2D). In both sexes the decreases were closely associated with elevations in corticosteroid levels.

Sham-castrated males and females. As was the case with castrated males, the initial resting levels of plasma corticosterone in sham-operated males were relatively high (Fig. 3A). The subsequent pattern of changes observed in this group included a secondary peak in corticosterone at 1900. The variations found in sham-ovariectomized females were quantitatively similar to those observed in intact females, and a conspicuous peak appeared at 1500.

Figure 3B summarizes the 24-hr pattern of adrenal concentrations of corticosteroid in sham-operated animals. The males demonstrated a pattern that was similar temporally and quantitatively to that of intact males. The females showed a marked peak at 1500 which was somewhat higher than that obtained in intact females.

Pituitaries of sham-castrated males, like those of intact males, demonstrated greatest ACTH concentrations in early morning (Fig. 3C). The tendency toward decreased concentrations of ACTH coincident with increased corticosteroid levels is similar to the pattern seen in intact males. The pattern of pituitary content of ACTH in sham-castrated females generally resembled that seen in intact females. Pituitaries of sham-castrated males and females, like those of intact males, demonstrated a slight decrease in ACTH during the period of highest corticosteroid levels, a pattern similar to that seen in intact males.
intact females: increasing amounts of ACTH were found in association with elevations in adrenal cortical activity. However, the two high levels at 0300 and 0700 were at variance with results obtained in intact animals.

Both sexes demonstrated a depression in the number of circulating leukocytes during or following elevations in corticosterone levels (Fig. 3D). A conspicuous sex difference in levels of leukocytes, more marked than in previous groups, was observed in sham-castrated rats.

Prepubertal males and females. As shown in Fig. 4A, variations in plasma corticosteroid in 30-day-old males were temporally and quantitatively similar to those observed in adult males. In contrast, the 24-hr pattern found in young females differed markedly from that of adult females. One obvious difference was the amplitude of the diurnal excursion. Whereas adult females demonstrated a peak of 59.90 ± 4.51 µg/100 ml, prepubertal females showed only 24.16 ± 6.65 µg/100 ml. Another difference was the appearance of a peak at 1100 instead of at 1900.

In prepubertal males, the pattern of adrenal concentration of corticosterone (Fig. 4B) included a peak at 1900, but otherwise it was similar to that observed in adult males. As in plasma, the 24-hr variations in the adrenals of young females were different from those of adult females. The absence of a more prominent peak at 1900 constituted the most conspicuous difference.

The variations in pituitary ACTH concentration in prepubertal rats (Fig. 4C) differed from those observed in adults. Young males had high levels in the early morning and there was a progressive decrease during the day as in adult males, but the value obtained at 2300 made the pattern discordant with that seen in mature males. The prepubertal females did not show the marked elevation in pituitary content of ACTH that was obtained in adult females.

Leukocyte counts for the 24-hr period in prepubertal males and females (Fig. 4D) followed patterns that were similar to those seen in the other groups, although there was an extended depression in males from 1900 to 0300.

To summarize between-group differences in resting levels of adrenal cortical secretion, mean corticosterone concentrations for 24 hr are shown in Table 1. The mean 24-hr concentrations of both plasma and adrenal corticosterone were highest in rats with mature ovaries, i.e., in intact and sham-castrated females. According to analyses of variance performed on individual samples over comparable 24-hr periods, concentrations of both plasma and adrenal corticosterone were significantly higher ($P < 0.001$) in intact and sham-castrated females than in the remaining groups. No significant differences were found between groups without mature ovaries.

Body and adrenal weights are also summarized in Table 1. Because of differences in body weights, relative adrenal weights were used. The adrenals of all females, intact, castrated, sham-castrated, and prepubertal, were heavier ($P < 0.01$) than those of corresponding groups of males. On a relative basis, the adrenals of both groups of 30-day-old rats were heavier ($P < 0.001$) than those of adults of the same sex. Whereas adrenals were heavier in castrated than in control or sham-castrated males ($P < 0.001$), ovariectomy in females was not associated with a change in adrenal weight.

Table 2 summarizes resting levels of plasma and adrenal corticosteroids of females with mature ovaries grouped according to stage of the estrous cycle at time of autopsy. To minimize the number of transitional types of vaginal smears, comparisons were limited to rats that were killed between 0300 and 1100. Plasma corticosteroid concentrations were higher during proestrus than during diestrus ($P < 0.001$). Adrenal corticosterone was in highest concentration during proestrus also, higher than during either estrus or diestrus ($P < 0.001$).
anesthesia. The effects of pentobarbital on diurnal elevations in plasma and adrenal corticosterone are shown in Table 3. Anesthetized females killed at either 1500 or 1930 had levels of plasma and adrenal corticosterone which were lower (P < 0.001) than resting levels observed in unanesthetized females at comparable times. In males, pentobarbital depressed (P < 0.05) corticosterone levels when administered at 1500; the levels in treated and untreated males were not different at 1930. In spite of anesthesia, sex differences in corticosterone concentrations were still conspicuous in plasma (P < 0.025) and adrenals (P < 0.05) of rats killed at 1930. No differences, however, were evident at 1500.

Plasma and adrenal corticosterone following a phase shift in lighting regimen. As illustrated in Fig. 5, intact rats of both sexes responded to the new pattern of illumination by demonstrating a shift of about 9 hr in the phase of the corticosteroid rhythm. The peaks in plasma and adrenal corticosterone appeared at approximately 0300, 0400, and 0500, instead of from 1500 to 1900. The basis for the absence of a sex difference in adrenal concentration of corticosterone at 0300 is unknown.

Blinded rats (Fig. 6) failed to demonstrate an appropriate shift in steroid patterns and showed a tendency to retain some of the phase characteristics of the rhythm that was imposed prior to enucleation. In males, however, the peaks in plasma and adrenals occurred somewhat earlier than those in intact males exposed to the original lighting schedule. The females demonstrated relatively high plasma and adrenal levels at the first 0700 sampling; however, the highest concentrations were still obtained in plasma and adrenals at 1500, in spite of the change in lighting.

As summarized in Fig. 7, ovariectomized females exposed to the altered lighting schedule showed the appropriate phase shift in the 24-hr corticosteroid pattern.
methods, however, complicate comparison of these results.

The results in castrated and prepubertal animals implicate the ovary in mechanisms responsible for the sex difference in the circadian rhythm of adrenal cortical function. Both castrated and prepubertal females failed to show the marked excursions of plasma and adrenal corticosterone that were present in intact and sham-castrated females. Comparison of the mean 24-hr corticosterone levels also points to the important role played by mature ovaries in the maintenance of the female pattern of resting adrenal cortical function; while there were no differences between levels found in ovariectomized and prepubertal females and those found in any of the groups of males, the values observed in intact and sham-castrated females were significantly higher than those of all other groups. Previous reports described decreased adrenal concentration of corticosterone following ovariectomy in rats (26) and mice (1), although no effect was observed on the corticosterone content of adrenal venous blood of rats by T'ellegdy et al. (33).

The lower resting levels of plasma and adrenal corticosterone that were observed following ovariectomy were manifested in the absence of a change in adrenal weight. Spayed females, like intact and sham-operated females, had adrenals which were heavier than those found in males. A similar dissociation between maintenance of weight and secretory activity was reported by Sakiz (27). Although in these studies no differences were observed in corticosteroid concentrations of intact and castrated males, increased adrenal secretory activity following removal of testes has been described (28).

Not only is the presence of mature ovaries an important factor in establishing the sex difference in resting adrenal cortical function, but cyclic variations in ovarian activity apparently lead to marked differences in corticosteroid secretion. The higher levels of plasma and adrenal corticosterone which were observed at proestrus suggest that estrogens may be primarily responsible for the female-type circadian pattern. Such an effect of estrogens would be consistent with Holzbauer's report of an estrogen-induced increase in adrenal cortical secretion under resting conditions (22). Exogenous estrogen is reported to influence adrenal cortical function in cats and dogs (6). According to Sakiz (28), rats show higher post-stress levels of adrenal corticosterone during estrus than during other stages of the reproductive cycle. In contrast to the results obtained in the present studies with unanesthetized rats, Guillemin et al. (13) and Telegdy and co-workers (33) did not find a correlation between corticosteroid levels and stages of the estrous cycle in anesthetized rats. The relationship between ovarian function and corticosteroid concentrations is probably responsible for some of the considerable variation that was noted within groups of females.

Although the method of assay of ACTH used in these studies precludes quantitative comparisons of pituitary ACTH concentrations between groups, it does permit comparison of relative changes in ACTH concentrations and the time-course of such changes. The variations in pituitary levels of ACTH, when considered with plasma adrenal corticosteroid data, suggest the presence of circadian rhythmicity in both content and release of ACTH. Furthermore, it appears that this rhythmicity differs in the two sexes. The tendency toward decreased ACTH content in pituitaries of males and ovariectomized females, coinciding with periods of increased adrenal cortical function, may reflect dominance of mechanisms involved in release of ACTH over those concerned with synthesis. Conversely, the increase in concentration of ACTH in pituitaries of females with mature ovaries during the diurnal elevation in corticosteroid levels suggests that processes of both synthesis and release are operant, but that the former predominates. These results may indicate an ovarian influence on mechanisms that regulate both synthesis and release of ACTH.

Gonadal influences on adrenal cortical secretory activity are apparently mediated at several levels. First, there may be direct effects on the adrenal as indicated by sex differences in responses to ACTH, both in vivo (4, 23, 24) and in vitro (29). Second, there is some evidence that gonadal hormones may influence central mech-
anisms that regulate the secretion of ACTH. Gemzell (9) reported that exogenous estrogen affected both the synthesis and release of ACTH. Also in this regard, preliminary reports indicate that female rats secrete more ACTH in response to stress than males do (2) and secretion of ACTH in response to stress is highest under conditions when endogenous estrogen is maximal (1). Finally, the gonads may influence pituitary-adrenal function indirectly through effects on mechanisms responsible for inactivation, through hepatic enzyme systems (10, 23) or protein binding (30, 32), of corticosteroids that otherwise participate in the negative-feedback control of ACTH release. In the present studies, the suggestion of a sex difference in 24-hr patterns of pituitary content and the differences observed in ovariectomized and prepubertal females are consistent with an ovarian influence on synthesis and release of ACTH, but furnish no information as to the mechanisms involved.

The results obtained in rats subjected to a phase shift in lighting regimen are in agreement with reports of the synchronizing effect of light rhythms on the 24-hr corticosteroid pattern in mice (16). Thus, it appears that the rhythm of environmental illumination exerts a phasing influence on the pituitary-adrenal axis which is similar to that described for the pituitary-gonadal system (3, 21). Apparently the eyes are important for synchronization of the adrenal cycle by light because the appropriate shift was not noted in blinded animals. The indication of some maintenance of a previously imposed rhythm in rats without eyes is consistent with the report that blinded or dark-exposed rats maintained rhythms of motor activity and estrous cycles that were previously established (3). The "blunting" of the corticosterone peaks in optic-enucleated rats may represent some randomization of adrenal cycles due to the absence of a precise synchronizing influence. The response of ovariectomized females to the changed lighting suggests that despite the marked influence of ovarian function on adrenal secretion, light affects the hypothalamo-pituitary-adrenal axis without mediation of the ovaries.

The possibility exists that the shift in the rhythm of corticosteroids in rats with intact visual pathways was due to a change in patterns of motor activity rather than a more direct influence of light on the pituitary-adrenal system. However, the former possibility may not be the case because Halberg and co-workers (20) reported that in mice the 24-hr changes in corticosterone concentrations precede those occurring in motor activity, and it has been suggested that the adrenal rhythm is preparatory to cyclic motor activity (18).

The shift in the 24-hr pattern of corticosteroids that followed a change in phase of environmental lighting indicates that the nervous system, probably via the hypothalamic-pituitary axis, is involved in mechanisms underlying the circadian rhythm of adrenal function. Suppression of the diurnal elevation in corticosteroid levels in male and female rats following the administration of pentobarbital is compatible with the suggested participation of the nervous system in processes leading to the marked daily excursion in adrenal cortical secretion.

The evidence for a 24-hr rhythm in levels of circulating leukocytes observed in the present studies is in agreement with previous findings in rats (14). This apparent rhythmicity probably has its basis in cyclic variations in adrenal cortical secretory activity since the relationship between circulating corticosteroids and leukocytes is well documented (11).

Of interest in regard to problems of 24-hr rhythms of endocrine activities are the several similarities that exist between processes leading to cyclic ovarian activity in the rat and those that are involved in the 24-hr variations in adrenal cortical activity. As appears to be the case with ACTH release under resting conditions, cyclic gonadotropin secretion leading to ovulation has characteristics of a 24-hr rhythmicity (7), is subject to regulation by environmental lighting rhythms perceived through the eyes (3, 21), is absent or diminished in prepubertal rats, and involves pentobarbital-sensitive neural mechanisms that are active in the afternoon under the lighting conditions used in these experiments (8). These similarities offer a basis for postulating that closely related neuroendocrine mechanisms are involved in the cyclic release of ACTH and gonadotropins.

![Graph](http://ajplegacy.physiology.org/)

**Fig. 6.** Twenty-four-hour patterns of resting levels of plasma (A) and adrenal (B) corticosterone in blinded male and female rats 3 weeks following a 9-hr shift in lighting regimen.
REFERENCES