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Temporal Variations in Reproductive Hormones in the Immature Male Rat (39401)

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Rhythmical changes in serum levels of hormones are well known (1). In the adult male rat a daily maximum in luteinizing hormone (LH) and prolactin has been reported to occur at about the middle of the dark period (2); another study showed the peak in prolactin at the beginning of the dark period (3). In the human male the highest serum levels of LH (4) and testosterone (5) have been found late in the dark or early in the light period. A daily peak in serum testosterone for the adult male rat was found at 0300, which was also late in the dark period (6). On the other hand, rhythmical changes in serum follicle stimulating hormone (FSH) have been difficult to establish; one study (7) in the male rat failed to show a rhythm while another (8) reported a peak in serum FSH at 0400 or 2 hr before the start of a daily 14-hr light period.

In contrast to the situation in adults, much less information is available on the temporal changes in hormones of immature animals. The concept is generally accepted that rhythms develop with age and become established only at about the time of puberty (9). Prominent nocturnal increases in serum LH (10) and testosterone (11) have been reported for the pubertal human male. In the prepubertal male rat (35 day) rhythms in FSH or LH were not found (8). The lack of information regarding variations in hormonal levels in the post weaning male rat prompted the present study; the results show distinct changes with time.

Materials and methods. Male rats of the Holtzman strain were used. They were housed in temperature (24 ± 2°) and light (14 hr light: 0600–2000) controlled quarters with free access to food and tap water. Cages were opened and each animal handled daily prior to the beginning of the study. A group of five animals was decapitated every 4 hr from 0800 on the twenty-fifth day until 1600 on the twenty-sixth day of age. Decapitation was done as soon as possible (5–15 sec) after the animal was removed from the cage. Since stress may alter hormonal patterns (2), efforts were made to minimize disturbance during this procedure.

Serum was separated by centrifugation (1500 *g* for 15 min) after the blood had clotted at room temperature; it was stored at −17°. Assays were performed in the following sequence: (i) LH(homologous system); (ii) FSH; (iii) testosterone; (iv) androstenedione(androst-4-ene-3,17 dione); (v) LH (heterologous system); (vi) 20α-dihydroprogesterone (20α-hydroxypregn-4-en-3-one) (20αDHP); and (vii) progesterone. If there was insufficient volume of serum for the next determination the sample was dropped from the study.

LH and FSH were measured by a double antibody radioimmunoassay using materials supplied by the NIAMDD-Rat Pituitary Program. LH was also assayed using anti-ovine LH (prepared by Dr. Gordon Niswender, Colorado State University) and iodinated ovine LH (NIAMDD). The details of the assay procedures have been published (12). The results of all assays were expressed in terms of rat standards (RP-1 LH; RP-1 FSH).

The steroids were also measured by radioimmunoassay. Anti-testosterone and anti-progesterone were prepared in this laboratory and their characteristics in assays have been reported (13). Anti-androstenedione was a gift of Drs. H. Lindner and S. Bauminger of the Weizmann Institute (Rehovot, Israel); its use in assays of this steroid have been published (14). Anti-20αDHP was supplied by Drs. C. N. Pang and J. Hilliard (University of California, Los Angeles). The 20αDHP was attached through the carbon-3 position to bovine serum albumin for an antigen. The only steroid which gave appreciable cross-reactivity (>1%) with the antiserum was 20βDHP (42%).

Tritiated steroids were purchased from New England Nuclear Corp (Boston, Mass). Specific activities were: testosterone 45 Ci/mmole; androstenedione 48 Ci/mmole; progesterone 50.3 Ci/mmole; 20 DHP 50.3 Ci/mmole. Steroid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were dissolved in methanol.

The steroid assays were performed in phosphate buffered saline (0.1 *M*, pH 7.0). Serum (25–50 μl) was diluted with 1 ml of distilled water and extracted once with 3 volumes of freshly opened diethyl ether. Extraction efficiency was evaluated by extraction of 5000 cpm of steroid from 1 ml of water. The ether extracts were taken to dryness and redissolved in 0.1 ml of buffer containing 0.1% gelatin. Sufficient antibody to bind 40–55% of the labeled steroid was added and the solution incubated at room temperature for 30 min. After addition of 15,000 dpm of labeled steroid, the incubation was continued overnight at 4°. Free and antibody-bound steroids were separated by dextran-coated charcoal and centrifugation for 10 min at 1500 *g*. A 0.5-ml aliquot of the supernatant was placed in a scintillation vial with 5 ml of Bray's solution and counted for 10 min in a Packard scintillation spectrometer (35% efficiency for tritium).

Standard curves for gonadotropin and steroid assays were linearized by logit and log dose transformations; unknowns were extrapolated after linear regression analysis. Samples were randomized and analyzed in duplicate for a particular hormone in a single radioimmunoassay. Intraassay coefficients of variation between duplicates did not exceed 10%. Values below the sensitivity of the assay were not included in statistical calculations. Data were analyzed by one-way analysis of variance and by Duncan's new multiple range test (15), with a probability of less than 5% considered statistically significant.

Results and discussion. The results of the study are shown in Fig. 1. Serum FSH concentrations changed significantly with time [$F(8,29) = 2.66$, $P < 0.05$]. It was high in the morning (0800), lower during the remainder of the light period (1200–1600), and then steadily increased to a high point near the end of the dark period (0400). A very similar pattern was found previously in adult males of the same strain housed under the same conditions (8). Serum testosterone [$F(8,35) = 3.09$, $P < 0.01$] and androstenedione [$F(8, 35) = 3.39$, $P < 0.01$] also had significant variations with time; the androgens were correlated with each other ($r = 0.95$, $P < 0.01$).

Testosterone showed the same degree of variation and the same changes with time as has been reported for the adult male rat (6). In the latter study serial bleedings from animals under ether anesthesia were used: ether is a stress that can alter endocrine rhythms (2). Although the serum androgens followed the same general pattern of changes as did FSH, comparison of individual serum samples shows that there is not a significant correlation (FSH:testosterone, $r = 0.27$) (FSH:androstenedione, $r = 0.26$). However, the recent finding that highly purified FSH is as effective as LH in raising serum testosterone in hypophysectomized male rats (16) indicates that we must consider the possibility that the FSH was the stimulus for the increases in androgen.

Serum LH[$F(8,33) = 1.17$, NS], progesterone [$F(8,25) = 1.74$, NS], and 20αDHP [$F(7,27) = 1.09$, NS] did not significantly vary with time. LH was highest at 0800 and 0400 but large differences between animals within groups prevented statistical significance. The results for LH were confirmed (data not shown) in a heterologous ovine radioimmunoassay system which is more sensitive than the homologous assay. Comparison of LH levels with those of testosterone in the same samples failed to show a significant correlation ($r = 0.15$), but LH and FSH are correlated ($r = 0.51$, $P < 0.01$).

The demonstration of a distinct rhythm in serum androgens without significant changes in LH suggests lack of a relationship. However, we measured a rapidly disappearing gonadotropin at infrequent intervals and therefore short periods of increased output of LH by the pituitary could easily be missed. There is the possibility that rhythmical changes in Leydig cell steroidogenic

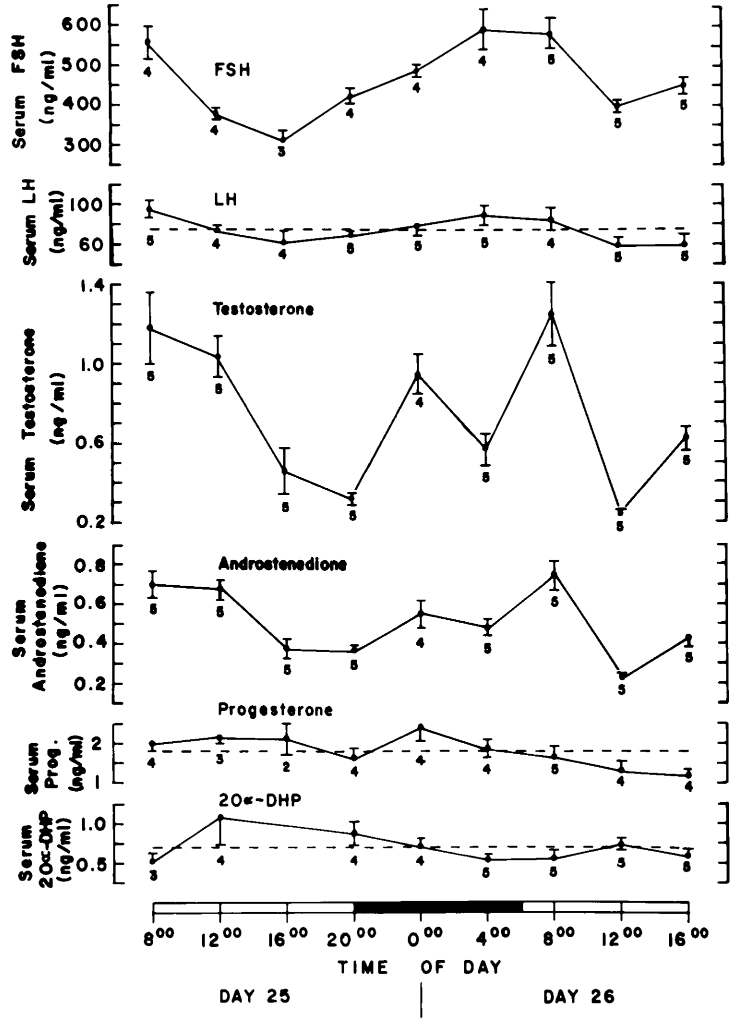

FIG. 1. Variations with time in serum gonadotropin and steroid concentrations in male rats. Groups were decapitated at 4-hr intervals on the twenty-fifth and twenty-sixth days of life. Vertical lines indicate standard errors for the number of observations indicated. The dashed line denotes the grand mean when variations with time were insignificant. Shaded area indicates daily dark period.

function occur independently from changes in gonadotropin. Previous studies using hypophysectomized immature male rats treated with LH and prolactin suggested that the testes were more sensitive to gonadotropic stimulation during the dark period (17). A similar suggestion has been proposed to account for the diurnal variation in testosterone in the human (11). Preliminary experiments (D. C. Johnson and H. C. Cheng, unpublished) have shown that increased testosterone levels are maintained longer in hypophysectomized males given LH and prolactin in the dark period compared to those injected in the light period; the magnitude of the increase in androgen was not time dependent, only the duration. These results could also be interpreted as indicating fluctuations in the rate of destruction of hormone, rather than in the rate of secretion. This aspect has been inadequately studied but in the human at least the metabolic clearance rate of testosterone does not change with time (5).

The significance of the changes with time found in the present study are not clear. The pattern of serum concentrations needs to be followed for a much longer time in order to establish whether a true circadian rhythm (20–28 hr) is involved. These data do clearly demonstrate, however, that studies of gonadotropins and androgens in immature male rats must consider temporal changes. The absolute values for the serum androgens in the immature rats were relatively low but the temporal fluctuations for testosterone represent a fivefold and for androstenedione a threefold variation in mean concentrations.

Summary. Radioimmunoassays were used to measure serum levels of LH, FSH, testosterone, androstenedione, progesterone, and 20αdihydroprogesterone in immature (25–26 day) male rats decapitated at various times of the day. Only FSH, testosterone, and androstenedione showed a significant variation with time; the maximal values occurred late in the dark and early in the light period.

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