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# Melatonin secretion decreases during the proestrous stage of the rat estrous cycle

(norepinephrine/rhythms/chromatography/radioimmunoassay)

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**ABSTRACT** **Urine was collected from rats during 12 consecutive daily dark periods and assayed for melatonin and norepinephrine; the phase of the vaginal estrous cycle associated with each urine sample was determined from daily vaginal smears. The proestrous phase of the estrous cycle was consistently associated with significant reductions in the excretions of both compounds. The level of melatonin in any urine sample tended to vary as a function of its norepinephrine content; however, the slope of the curve relating these two compounds in metestrous-diestrous samples differed from that for proestrous-estrous specimens. This difference suggests that factors other than the catecholamine (e.g., gonadal hormones) also affect melatonin secretion. Oophorectomy elevated the melatonin concentration of serum but not that of the pineal; this rise was suppressed by the administration of estrogen plus progesterone. The fate of circulating melatonin (as indicated by the proportion of an exogenous dose excreted into the urine) was not affected by the state of the estrous cycle.**

The administration of melatonin, an indolic hormone synthesized in the mammalian pineal organ (1–4), inhibits the growth and functional activity of the rat ovary (4–6); for example, it delays vaginal opening, reduces ovary weight, depresses the incidence of vaginal smears showing estrous cytology, and suppresses the secretion of luteinizing hormone (7, 8).

The rate at which pineal organs synthesize (9–11) and secrete (12–14) melatonin has been shown to vary with a characteristic 24-hr rhythm that is well synchronized with the environmental photoperiod. Melatonin production and release are, in all species examined, greatest during the daily dark period, falling precipitously with the onset of the light period. Normally this circadian rhythm is synchronized by nerve impulses generated by light acting on the retinas and transmitted to the pineal via norepinephrine released from its sympathetic nerves (15). Several of the enzymes required for melatonin biosynthesis have also been shown to be influenced by circulating hormones and to exhibit rhythmic changes that parallel the estrous cycle (16, 17). Studies described in this report show that melatonin secretion in untreated adult rats also varies with the phase of the estrous cycle and that it is diminished by gonadal steroid hormones.

## MATERIALS AND METHODS

Female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), caged in groups of three to five, were maintained at temperatures of 23 ± 2° in a room lit by Vita-Lite (Duro-Test Corp., North Bergen, NJ) at an intensity of 300 $\mu W/cm^2$ at the level of the animals; lights were on between 8 a.m. and 8 p.m. The animals were left undisturbed for at least 7 days before use in an experiment. In studies on estrous rhythms, vaginal smears were performed daily, and only rats showing at least three consecutive 4-day cycles were used. In studies on urinary melatonin and norepinephrine, urine was collected from 60-day-old rats between 8 p.m. and 8 a.m. [the period during which at least 80% of the total daily melatonin output is secreted (14)] for at least 12 consecutive days; the rats were housed individually in metabolic cages (22.5 × 22.5 × 27.5 cm; Acme Metal Products, Chicago, IL). In studies on the fate of circulating melatonin, 60-day-old rats received a melatonin injection (250 ng in corn oil, subcutaneously) between 7:30 and 8 p.m. daily for 4 days, and their overnight urine specimens were collected for melatonin assay; daily vaginal smears were also taken and examined. In studies on the effects of castration and exogenous gonadal hormones (estrogen and progesterone), we used 100-day-old rats that had been bilaterally ovariectomized at 40 days of age. The animals were decapitated at 2 a.m. (i.e., 6 hr after the onset of darkness); their pineals and serum samples, which were obtained from trunk blood, were taken for melatonin assays.

Trunk blood from individual animals was collected in 16 × 100 mm glass tubes; sera were separated by centrifugation. Immediately after the rats were decapitated, their heads were placed in an ice slurry, and the pineals were removed and frozen in glass homogenizers on dry ice. Each 12-hr urine sample was collected in a 25-ml flask containing 1 ml of 1 M HCl and 20 mg of sodium metabisulfate, its volume was measured, and it was centrifuged at 3000 × *g* for 10 min. The resulting supernatant fluid was frozen and stored at −20° prior to assay.

The melatonin in urine was extracted into an equal volume of chloroform and isolated by use of a unidimensional multiple-chromatographic procedure described below. After centrifugation at 500 × *g*, the aqueous phase was transferred to a 16 × 100 mm glass tube and its norepinephrine (and epinephrine) content was extracted and assayed. The remaining organic phase was washed successively with 4 ml of 1% sodium bicarbonate solution and 4 ml of deionized water; it was evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 100 μl of absolute ethanol and applied to a thin-layer chromatographic plate (20 × 20 cm silica gel-coated Quantum Linear-Q plate; Quantum Industries, Fairfield, NJ) that had been treated with ethanol/ascorbate solution (1 g of ascorbate per 100 ml of absolute ethanol). The plates were developed by ascending multiple chromatography: we developed each plate to 60 mm, and then dried and developed it again a total of eight times within a period of approximately 3 hr, using pure chloroform as solvent. A 5-mm segment corresponding to the location of authentic melatonin (chromatographed concurrently) was eluted from each plate with 1 ml of absolute methanol. The eluate was filtered through a phase-separating filter (Brinkman Instruments Inc., Westbury, NY) and evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 1 ml of 10 mM Tris buffer (pH 7.4), and du-

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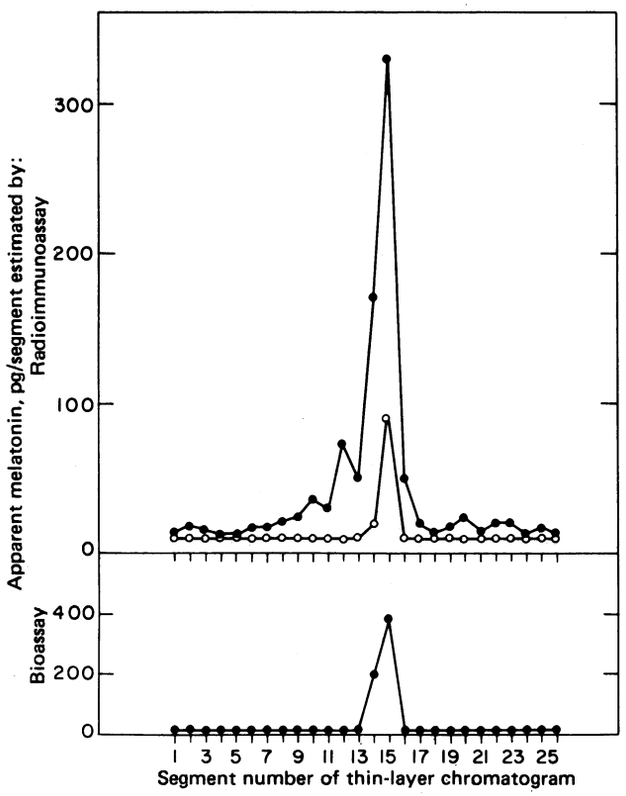


FIG. 1. Programmed-multiple-development thin-layer chromatographic resolution of constituents of rat urine extract, evaluated by both bioassay and radioimmunoassay. Urine extracts were chromatographed on silica gel thin-layer plates with pure chloroform as developing solvent. Five-millimeter segments of the developed chromatogram were eluted and assayed for melatonin. Authentic melatonin (chromatographed concurrently) was used for a chromatographic reference; it was assayed similarly by radioimmunoassay. ●, Urine extract; ○, authentic melatonin.

plicate aliquots (100–400 μl) were subjected to radioimmunoassay (18). The recovery of added melatonin (1 ng) by this procedure was 70–80%.

The melatonin in serum was extracted into 3 volumes of chloroform; this organic extract was washed successively with sodium bicarbonate solution and water, after which the organic phase was evaporated to dryness, and the dried residue was dissolved in 100 μl of absolute ethanol. The melatonin was then isolated by use of the unidimensional multiple-chromatographic method and assayed by radioimmunoassay as described above. The recovery af added melatonin from sera also averaged 70–80%.

The melatonin in pineals was extracted into chloroform from 0.1 M HCl homogenates; after being washed similarly, it was reextracted into Tris buffer in the presence of excess *n*-heptane and subjected to radioimmunoassay as described previously (14).

From the urine samples that had previously been subjected to extraction (of melatonin) with chloroform, norepinephrine and epinephrine were adsorbed onto alumina (19). The acetic acid eluates were analyzed fluorimetrically for the catecholamines (20).

The radioimmunoassay for melatonin was performed as described by Levine and Riceberg (18); rabbit anti-melatonin globulin was generously provided by Lawrence Levine (Brandeis University, Waltham, MA). The sensitivity of this assay was $5 \times 10^{-2}$ pmol per sample; the intra-assay and inter-assay coefficients of variation for 100-pg samples were 3 and 4%, respectively. We also estimated the melatonin contents of some extracts by using a bioassay based on the dermal melanophore response of larval anurans to the melatonin in their bathing medium (21).

Pooled extracts containing sufficient quantities of melatonin for both radioimmunoassay and bioassay were also subjected to an authentication procedure that used an open-chamber programmed-multiple-development device, which accomplishes multiple development through a clock-operated rack-and-pinion mechanism. In accordance with a predetermined program, it allows the solvent (e.g., chloroform) front to advance repeatedly to 120 mm. Between periods of development, the thin-layer chromatographic plate and its chamber are automatically withdrawn from the developing solvent and the solvent is allowed to evaporate from the silica gel surface. The system affords a distinctive resolution of melatonin; a mixture of melatonin and *N*-acetyltryptamine (compounds virtually inseparable by simple chromatographic methods) applied to a thin-layer chromatographic plate and subjected to this procedure is resolved in 24 hr as two discrete bands 10 mm apart. When the constituents of a urine extract were distributed on a chromatogram in this manner and 5-mm segments were eluted and submitted to both radioimmunoassay and bioassay, virtually all of the material detectable by bioassay, and most detected by immunoassay, exhibited mobility identical to that of authentic melatonin (Fig. 1).

Data were evaluated by analysis of variance and covariance for repeated measurements, linear correlation and regression analysis, Bartlett's test for homogeneity of variances, and *t*-test; these analyses were performed by use of an IBM S/370 model 168 computer and the Biomedical Computer Programs (revised in August, 1976) developed at the Health Science Computing Facility, U.C.L.A., under N.I.H. Special Research Resources Grant PR-3 (22).

## RESULTS

### Variations in urinary melatonin and norepinephrine contents with the estrous cycle

In each of two experiments, individual nocturnal urine samples were collected for 12 consecutive days from six rats known to undergo regular 4-day vaginal estrous cycles. Vaginal smears taken daily and concurrently were identified as proestrous, estrous, metestrous, or diestrous, and data for each phase for each animal were pooled. (The data for each phase usually consisted of three individual samples collected on three different days.) Highly significant phasic variations in melatonin excretion were consistently observed ($P < 0.001$ by analysis of variance); Fig. 2*A* shows typical estrous patterns. A 30–50% fall in melatonin excretion was consistently observed for each animal between metestrus or diestrus and proestrus (Fig. 2*B*; $P < 0.001$ by *t*-test); estrous values were intermediate. The reduction in melatonin excretion during proestrus (Fig. 2) was consistent with the reduction in melatonin-synthesizing activity noted previously (16).

The levels of norepinephrine (but not epinephrine) in the urine of individual rats exhibited similar phasic variations ($P < 0.05$ by analysis of variance); paralleling the reduction in melatonin excretion, norepinephrine levels were also lowest on the night of proestrus (Fig. 2*B*; $P < 0.005$ compared with metestrus or $P < 0.01$ compared with diestrus, by *t*-test).

The relationship between the melatonin and norepinephrine contents of individual samples was apparently linear, i.e., the slope of the line generated from data of urine collected during all phases was highly significant at $P < 0.001$, by analysis of

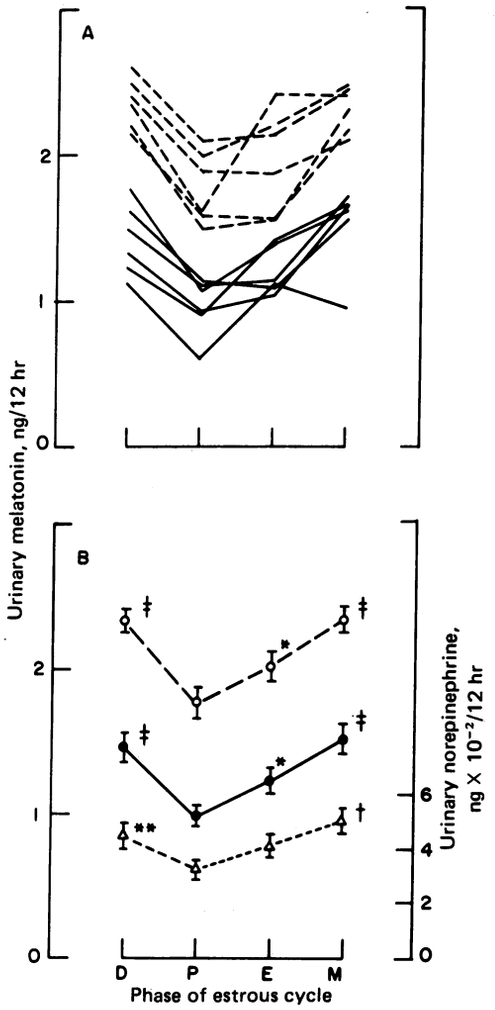


FIG. 2. Cyclic variations in melatonin and norepinephrine in rat urine, which parallel the estrous cycle. D, diestrus; P, proestrus; E, estrus; and M, metestrus. Urine specimens were obtained from 12 individual 60- to 76-day-old rats during the dark period (8 p.m.–8 a.m.) of 12 consecutive days (three full estrous cycles); vaginal smears were also taken daily. (*A*) Each point represents the mean melatonin content of three overnight urine specimens collected during a given phase of the individual rat's estrous cycle. Group I rats (broken lines, $n = 6$) were studied in May, 1976; group II rats (solid lines, $n = 6$) were studied in May, 1977. (*B*) Mean urinary melatonin contents were obtained by pooling data from the six animals in each experimental group (○ - - - ○, group I; ●—●, group II). The phasic variations in the mean urinary norepinephrine contents were similarly estimated from data on the six rats in group II (△ - - - △). Each point represents the mean melatonin (or norepinephrine) excretion (in ng/12 hr) ± SEM. * $P < 0.05$; ** $P < 0.01$, † $P < 0.005$, and ‡ $P < 0.001$ differ from the concentrations of melatonin (or norepinephrine) in samples collected during the dark period of the proestrous phase.

variance. Its correlation coefficient (0.430) was significantly different from zero at $P < 0.01$ (Fig. 3). If the data were subdivided, and those from metestrus-diestrus and proestrus-estrus plotted separately, it was apparent that urinary melatonin and norepinephrine were significantly correlated only for the former (Fig. 3; slope = 1.19; $P < 0.01$ by analysis of variance; correlation coefficient = 0.434, $P < 0.01$). The slope for the least-squares line representing proestrous-estrous phases was insignificant (Fig. 3; $P > 0.1$ by analysis of variance). This finding suggests that factors other than norepinephrine—presumably circulating gonadal steroids—significantly affect melatonin secretion during these phases.

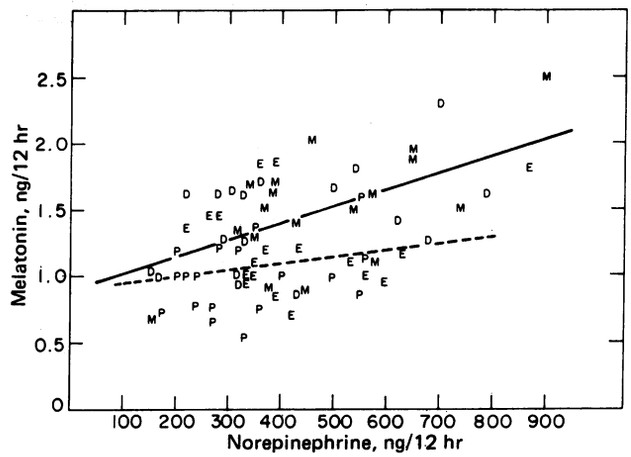


FIG. 3. Relationship between melatonin and norepinephrine contents estimated in urine collected during the 12-hr daily dark period from six individual 60- to 76-day-old rats. Data were analyzed by use of a simple linear correlation and regression analysis. The relationship between melatonin and norepinephrine was linear during the metestrous (M)-diestrous (D) phase (solid line); its slope (1.19) was statistically significant ($P < 0.01$). The correlation coefficient (0.434) was also significantly different from zero ($P < 0.01$). In contrast, the slope of the regression curve produced from data for the proestrous (P) and estrous (E) samples (broken line) was insignificant ($P > 0.1$).

## Effects of oophorectomy, estrogen, and progesterone on melatonin levels in rat pineal and serum

Nocturnal sera from 100-day-old rats, oophorectomized 60 days earlier, contained significantly higher melatonin concentrations than those from sham-operated animals ($P < 0.01$; Fig. 4). Administration of either 17-β-estradiol benzoate (50 μg/kg, subcutaneously, 55, 31, and 7 hr prior to decapitation) or progesterone (1 mg/kp, subcutaneously, 7 hr prior to decapitation) partially suppressed this elevation; administration of both

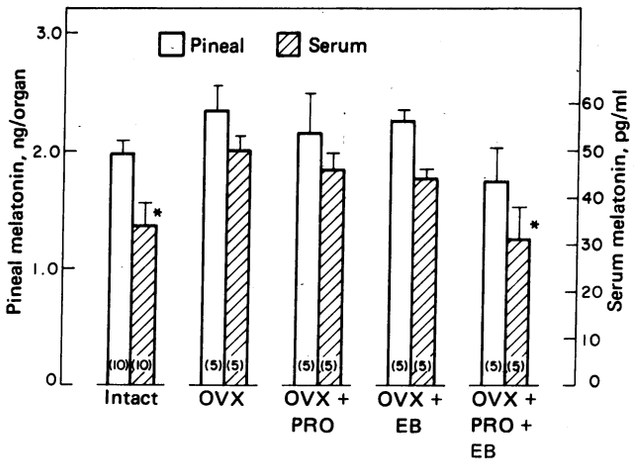


FIG. 4. Effects of oophorectomy, estrogen, and progesterone on melatonin levels in rat pineal and serum. Tissues were taken from 100-day-old rats that were either ovariectomized or left intact at 40 days of age. Some of the oophorectomized rats received 17-β-estradiol benzoate (50 μg/kg, subcutaneously, 55, 31, and 7 hr prior to decapitation) and/or progesterone (1 mg/kg, subcutaneously, 7 hr prior to decapitation). Each bar represents the mean melatonin levels in pineal or serum samples collected from 5–10 animals (in parentheses). Vertical lines indicate standard errors of the means. * $P < 0.01$ differs from melatonin levels in samples from oophorectomized animals treated with vehicle (corn oil) alone. OVX, ovariectomized; EB, 17-β-estradiol benzoate; PRO, progesterone.

ovarian hormones fully suppressed it ($P < 0.01$; Fig. 4). Neither oophorectomy nor gonodal steroid administration significantly altered pineal melatonin levels.

### Effect of phase of estrous cycle on the excretion of a given dose of exogenous melatonin

To determine whether the fall in urinary melatonin occurring during proestrus represented changes in the metabolism or renal clearance of the circulating indole (as opposed to a reduction in its rate of secretion), we gave animals a fixed dose of melatonin (250 ng/day in corn oil, subcutaneously) at various phases of the estrous cycle and determined its effect on urinary melatonin levels. Total melatonin excretion among groups of rats receiving a single dose of melatonin on one of the 4 days of the estrous cycle were: proestrus, 3.20 ± 0.30 ng/12 hr (mean ± SEM); estrus, 3.24 ± 0.30 ng/12 hr; metestrus, 3.43 ± 0.35 ng/12 hr; and diestrus, 3.47 ± 0.42 ng/12 hr. If urinary melatonin levels resulting from the endogenous hormone (Fig. 2) are subtracted from these values, there are no day-related differences in the increments of urinary melatonin caused by this melatonin dose. Hence, it seems likely that the cyclic changes in urinary melatonin (Fig. 2) reflect variations in the hormone's secretion and not in its peripheral metabolism or renal clearance.

## DISCUSSION

The recent development of assays for melatonin in body fluids allows direct investigations of the factors controlling melatonin secretion and the amounts of the hormone acting on peripheral tissues (12–14; 23–25). Data obtained by these assays have previously affirmed speculations about pineal secretory activity (e.g., as a function of time of day) based on *in vitro* assays of melatonin-forming enzymes. The present data affirm yet another such speculation (16), specifically, that melatonin secretion is suppressed by ovarian steroids and tends to be lowest at the phase of the estrous cycle (proestrus) when the secretion of these steroids is greatest (16).

The rate of melatonin excretion during the 12 hr of the daily dark period clearly varies synchronously with the vaginal estrous cycle (Fig. 2). It is lowest during the proestrous phase, the time previously observed to coincide with minimal activities of the pineal enzymes that form melatonin and cyclic AMP (16, 26). This reduction during proestrus is probably caused, in part, by a decrease in the amount of the sympathetic neurotransmitter norepinephrine acting on the pineal (Fig. 2). Not all of the estrous variation in melatonin excretion can, however, be attributed to norepinephrine effects (e.g., during proestrus and estrus; Fig. 3); some variation may result from direct inhibitory actions of ovarian steroids on the pineal (Fig. 4). The reduction in urinary norepinephrine coincident with the proestrous and estrous phases of the estrous cycle is similar to that observed previously (27, 28) in plasma norepinephrine concentrations.

The experiments on the effects of exogenous melatonin on levels of melatonin in urine affirm that most of the indole in the bloodstream is rapidly metabolized (29) such that less than 1% escapes into the urine. These experiments also suggest that estrous variations in urinary melatonin levels reflect real differences in the secretion of this pineal hormone.

Most previous studies have emphasized the inhibitory effect of melatonin on the gonads (4–8). Our present data indicate that the reverse relationship also holds, i.e., that gonadal steroids inhibit melatonin secretion. In rats at least, melatonin and the gonadal steroids may be components of a complex feedback mechanism through which the pineal and ovarian hormones each inhibit the secretion of the other. The suppression of melatonin secretion by estrogen and progesterone may serve to slow the decrease in estrogen secretion that would otherwise occur when plasma estrogen levels start to rise.

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