Circadian and Estrous Rhythms in Pineal Melatonin and 5-Hydroxy Indole-3-Acetic Acid.* (29014)

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The pineal organ of the rat, as well as that of other mammals insofar as known, contains a high concentration of serotonin (5-hydroxytryptamine) (= 5-HT)(1). Smaller quantities of related 5-hydroxy and 5-methoxy indoles are also produced and contained by the pineal(1,2), and may have, in part, greater pineal specificity as metabolic intermediates or possibly as secretory products. The most pineal-specific of these, according to current evidence, is melatonin (N-acetyl-5-methoxytryptamine), derived within the pineal by N-acetylation of serotonin and O-methylation of the product(3,4). It has been suggested recently, but not without conflicting evidence, that melatonin may be a pineal hormone in mammals(5-9). In addition to the pineal-specific pathway from serotonin to melatonin, the occurrence of a less restricted metabolic route, from serotonin to 5-hydroxy indole-3-acetic acid, has been suggested(10,11).

The serotonin content of the rat pineal has been shown to follow a 9-fold daily (circadian) rhythm which is triggered by darkness and influenced by light, and, which is, to a slight but significant degree, modified by, or shows fluctuations correlated with, the estrous cycle(12). The suggestion made in this previous work, that the nocturnal reduction in pineal serotonin represents a triggered release of melatonin-synthesizing activity, is supported by the results reported herein. Circadian and estrous rhythms in pineal melatonin and 5-hydroxy indole-3-acetic acid are demonstrated for the first time and are discussed in relation to additional evidence for the nature and timing of the pineal's metabolic and possible secretory activities.

Methods and materials. Long-Evans rats born and raised within the same suite of artificially lighted and windowless rooms were used as previously(12). The estrous cycles of the females were determined from daily vaginal smears taken at 13:00-15:00, and those animals departing from a cycle 4 days in length were discarded. At sacrifice, animals were quickly decapitated with minimal trauma and the pineals removed and freed of adjacent tissue and adhering blood. 5-HIAA was determined in samples containing 2 to 4 pineals each, from females 12 to 18 weeks old. Melatonin was determined in samples containing 4 to 6 pineals each, from males 12 to 13½ weeks old and females 18 to 28 weeks old. Melatonin-forming activity was determined *in vitro* according to the second procedure described previously(13), in which pairs of pineals were homogenized and incubated in 500 λ of an "improved substrate medium" to which 100 λ of serotonin was added just before incubation. Following incubation for 2 hours, at 37° and with agitation, each tube, including reagent blanks and standards, received 50 λ 1.0 N HCl and was chilled until extraction several hours later.

Pineal contents of 5-HIAA, melatonin and related indoles and incubated media contents of melatonin were extracted differentially and measured spectrophotofluorometrically as described previously(2,12). Use of even the best stocks of p-cymene for the extraction of melatonin required repeated washings to remove impurities(2).

Results. Pineal 5-HIAA in adult female rats follows a daily rhythm with a nocturnal minimum of about 4 ng/pineal and an early afternoon maximum of about 18 ng/pineal (Fig. 1). This rhythm resembles generally that followed by pineal serotonin content (12), but differs from it in timing in 3 significant features: (1) time of maximum content is significantly later; (2) drop to a nocturnal minimum is more rapid; and (3) nocturnal basal level is maintained for at least 5 hours before the start of the subsequent morning rise. The later rise and maximum

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point in 5-HIAA in comparison with serotonin are in harmony with the probability that the former is derived *in situ* from the latter. The rapid fall in pineal 5-HIAA on the beginning of darkness indicates that the fall in serotonin content at this time is probably not due to conversion to 5-HIAA.

Variation in pineal 5-HIAA content is least at times of maximal and minimal levels, and greatest during the middle of periods of transition (0730-0800, 1830-1900; note standard errors in Fig. 1), while variation in serotonin values(12) does not show such a pattern.

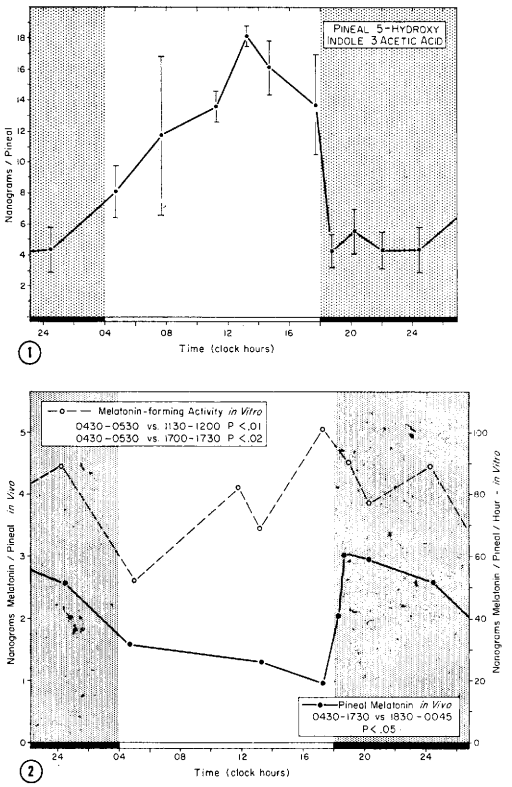

FIG. 1. Circadian rhythm in pineal 5-hydroxy indole-3-acetic acid in adult female rats. Means (dots) are plotted according to time of sampling in the daily periods of darkness (stippled) and light (clear). Vertical lines extend twice the standard error of the mean on each side of the means. Number of samples at each time (from left to right): 5, 38, 6, 6, 6, 5, 7, 5, 33, 6, 5.

FIG. 2. Circadian rhythm in pineal melatonin-forming activity in adult female rats, and melatonin content in adult males. Dots represent means of samples of the following sizes from left to right, melatonin-forming activity: 9, 18, 18, 10, 10, 17, 10, 9; melatonin content: 4, 8, 9, 8, 6, 8, 12, 4. P = probability based on "Student"–Fisher t.

Amount of readily extracted pineal melatonin is small in adult rats(2) as well as in other mammals studied(14), but is greater after the start of darkness than during the day (Fig. 2). Likewise, the pineal's ability to synthesize melatonin from serotonin is higher in the late afternoon and evening than earlier in the day (Fig. 2). The lack of a closer correlation between enzyme activity and onset of darkness, as well as the irregularities in the curve for this activity, are probably due at least in part to rupture of cell membranes and other artifactual conditions of the incubations. Nevertheless, the rhythm in melatonin content is in agreement with the hypothesis that melatonin is formed from serotonin at the start of darkness.

Pineal 5-HIAA content in early morning is significantly lower on the last day of the estrous cycle (diestrus) than on the first 2 days (Table I). This is the reverse of that reported for serotonin(12). The suggested trend in reduction in evening melatonin content during the estrous cycle (Table I) is weak statistically but is similar to the statistically significant trend in pineal serotonin at this same time(12).

Discussion. The specificity of the differential extractions and fluorometry used here has been analyzed previously(2) and appears to be satisfactory. In comparison with a previous estimate(15) of pineal melatonin content in the rat, 0.5 ng/pineal, my mean for the light period is about twice as great. This may be due to some loss occurring with the paper chromatography and bioassay procedures used in the earlier work(15), and slightly lesser specificity of the differential extraction technique employed here. The specificity of the former bioassay system, head and body melanophore contraction in larvae of *Xenopus*, has been examined in detail(16). Two compounds practically inactive in this bioassay(16) but capable of being extracted and measured in my procedure for melatonin are 5-methoxytryptamine and 5-methoxy gramine. However, the per cent recoveries (5 and 10%) of these are far less than that for

TABLE I. Mean Melatonin and 5-Hydroxy Indole-3-Acetic Acid Contents (ng/Pineal) of Pineals of Adult ♀ Long-Evans Rats. Daily photoperiod = 0400–1800. P = probability based on "Student"–Fisher t; S.E. = standard error of mean.

	5-Hydroxy indole-3-acetic acid at 0430–0500		2000–2030		Melatonin at 2030–2130	
Cycle day	No. of samples	Mean ± S.E.	No. of samples	Mean ± S.E.	No. of samples	Mean ± S.E.
1–proestrus	5	*7.26 ± .82	6	4.02 ± .71	14	3.7 ± 1.4
2	6	*7.02 ± .56	7	4.50 ± .67	15	3.2 ± 1.3
3	10	6.38 ± .50	6	3.27 ± .47	14	2.8 ± .9
4	7	*4.83 ± .30	6	3.25 ± .42	19	1.1 ± .7

* Day 4 *vs* day 1: P <.02; *vs* days 1 + 2: P <.01.

melatonin (73%) and their natural occurrence in pineal or other mammalian tissues has not been demonstrated.

The pineal indole precursors and intermediates, 5-hydroxytryptophan and N-acetylserotonin, are extracted from rat pineals in only very small and variable amounts(2) and recoveries of added standards of these to homogenates are sometimes poor.

Extensive studies were made of 5-methoxy indole acetic acid contents of male (11-13½ weeks old) rat pineals at the same times by methods similar to those used above and previously(2). The 53 samples gave a mean of 0.98 ng/pineal without significant evidence of any circadian rhythm.

The rat pineal's ability to form 5-methoxy indole acetic acid from 5-HIAA was tested with samples taken at different times and using a system similar to that used above and previously(13) for measuring melatonin-forming activity. What trace amounts, if any, of 5-methoxy indole acetic acid may have been formed were obscured in the extractive and fluorometric measurements(2) by the quantity of 5-HIAA.

The cellular localizations of pineal 5-HIAA and melatonin are not known, but pineal serotonin in the rat is claimed to be about equally distributed between parenchymal cells and nerve fibers(17,18).

The low pineal content of melatonin in comparison with serotonin raises the question whether melatonin is a quantitatively significant pineal product; the *in vivo* data suggest that a considerable amount of the serotonin is converted to 5-HIAA instead. It remains possible, however, that melatonin is either released (secreted?) or bound chemically nearly as rapidly as it is formed. Investigations of these possibilities as well as of related problems in pineal indole metabolism *in vivo* are aided by knowledge of the circadian changes and the probability that melatonin formation is greatest during darkness.

Summary. Differential extractions and fluorometry of pineal 5-hydroxy indole-3-acetic acid and melatonin in adult rats demonstrate circadian rhythms correlated with periods of light and darkness and possibly influenced to a lesser degree by the estrous cycle. Pineal 5-hydroxy indole acetic acid in females rises from a nocturnal basal level of 4 ng/pineal to an early afternoon maximum of 18 ng/pineal and falls sharply at the start of darkness. Pineal melatonin rises at the start of darkness from 1 to 3 ng/pineal; 5-methoxy indole acetic acid, averaging 0.98 ng/pineal, shows no circadian change. The specificity of the measurements and their implications for pineal indole metabolism and function are discussed.

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Metabolism of 9-Ethyl-6-Mercaptopurine by Humans.* (29015)

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It has been reported that 9-ethyl-6-mercaptopurine (9-E-6-MP) will produce remission when administered orally to chronic granulocytic leukemia patients(1). This drug is excreted in the urine of humans primarily as the S-glucuronide but a significant amount of the drug (10-20%) is excreted as a compound that has a U.V. spectrum identical with thiouric acid, (TUA)(2). However, the possibility has not been excluded that this compound is 9-ethyl-TUA since the spectrum of the 9-alkylated derivative would probably be identical with TUA. We have synethesized 9-E-6-MP labeled with C^{14} in the ethyl position (9-E-C^{14})-6-MP, administered it to patients and have also studied the effect of xanthine oxidase and nucleoside phosphorylase *in vitro* on 9-E-6-MP. These results are reported below.

Methods. 9-(E-C^{14})-6-MP was synthesized by published methods using C^{14}-ethylamine (1.85 mc/mmole) which was obtained from New England Nuclear(3,4). Xanthine oxidase (XO) was purchased from Worthington Enzymes. Inosine, hypoxanthine and 6-MP were obtained from Mann Research Laboratory, while 6-MP-ribotide was a generous gift from Dr. George Hitchings of Wellcome Research Laboratory. Spectra were obtained with a Beckman DU Spectrophotometer and enzyme reactions were carried out using the same instruments with attached thermospacer through which 37.5°C water was pumped. Ion exchange chromatography on Dowex-1 $\times$ 8 columns was performed as previously described(5). A Packard Tricarb liquid scintillation counter was used to assay samples for C^{14}.

Partially purified purine nucleoside phosphorylase (PNP) was prepared from rat liver as follows:

Rat liver was perfused with cold saline and 0.25 M sucrose followed by homogenization in 9 volumes of ice cold 0.25 M sucrose. The homogenate was fractionated by differential centrifugation by the method of Schneider and Hogeboom(6). The soluble supernatant obtained after removal of the microsomal fraction was adjusted to pH 5.2 with 10% acetic acid and left in an ice bath for one-half hour. It was then centrifuged at 8000 $\times$ *g*, the supernatant decanted from the precipitate, and the precipitate discarded. The supernatant which contained virtually all of the XO and PNP was dialyzed against pH 7.5 sodium phosphate buffer overnight. This solution was then fractionated with solid amon-

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