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Simultaneous Determination of *N*-Acetyltransferase Activity, Hydroxyindole-*O*-methyl-transferase Activity, and Melatonin Content in the Pineal Gland of the Syrian Hamster (41773)

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Abstract. The activities of serotonin *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) and the melatonin content were measured in Syrian hamster pineal glands at 2-hr intervals over a period of 24 hr. NAT and HIOMT are the two enzymes which catalyze the formation of melatonin from serotonin. The use of micromethods for determination of the enzyme activities allowed concurrent measurement of NAT and melatonin or HIOMT and melatonin in the same gland. HIOMT activity showed no significant diurnal rhythm whereas NAT activity and melatonin content exhibited distinct peak values late in the dark phase as described previously. Despite an apparent parallelism between the NAT activity rhythm and melatonin content, no correlation exists between these parameters in single pineal glands.

There are two enzymes involved in the formation of the pineal hormone melatonin from serotonin. First, serotonin-*N*-acetyltransferase (NAT) converts serotonin to *N*-acetylserotonin. In a second step, hydroxyindole-*O*-methyltransferase (HIOMT) catalyzes the synthesis of melatonin by *O*-methylating *N*-acetylserotonin. This metabolic pathway and its adrenergic regulation have been extensively studied in the rat pineal gland. The Syrian hamster pineal gland, however, shows different features in this pathway and the regulation of melatonin production is not fully understood (1, 2). Recently, we were able to determine the serotonin content in the Syrian hamster pineal gland and found a clear diurnal rhythm with high values in the morning and a nadir at midnight (3). In the same study, we were unable to detect *N*-acetylserotonin at any time point during the 24-hr period, indicating that *N*-acetylserotonin may be converted very rapidly to melatonin. The enzyme responsible for this conversion, i.e., HIOMT, has not been measured in the Syrian hamster over a complete 24-hr cycle. One aim of the present study was, therefore, to determine HIOMT activity throughout a 24-hr period. Originally, HIOMT was thought to be the rate-limiting step of melatonin production (4, 5), but to date many authors agree that NAT activity is an important regulatory event in melatonin synthesis in the rat pineal gland (6). NAT activity in the Syrian hamster shows a sharp peak late in the dark phase of the light/dark cycle and this pattern is reflected by the pineal melatonin concentration (7, 8). NAT activity is often used as an indicator of melatonin production and melatonin content in the pineal gland (9, 10). A second aim of this study was, therefore, to develop enzyme assays sensitive enough to measure both the enzymes and melatonin in the same gland; this would allow for a direct correlation of enzyme activities with the amount of melatonin formed.

Materials and Methods. A total of 96 male and 96 female Syrian hamsters (*Mesocricetus auratus*) were used in this study. They were purchased from Charles River Labs, Wilmington, Massachusetts, at the age of 35 days and housed in clear plastic cages in an air-conditioned (22 ± 2°C), windowless room under long photoperiod (LD 14:10, lights on at 0600 hr) for a period of 2 weeks before the experiment. Food and tap water were provided *ad libitum*. On the day of the experiment, groups of 16 hamsters (8 males + 8 females) were decapitated at 2-hr intervals over a period of 24 hr. Each pineal was quickly removed, immediately frozen on solid CO_2, and subsequently stored at −60°C. During the dark period, the pineal glands were collected with the aid of a dim red light. Eight glands (from

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4 males and 4 females) at each time point were assayed for NAT activity by radioenzymatic assay and melatonin content by radioimmunoassay; the other eight glands (again from 4 males and 4 females) were assayed for HIOMT by radioenzymatic assay and melatonin content by radioimmunoassay.

N-Acetyltransferase assay. NAT activity was measured using a combination of the methods of Deguchi and Axelrod (11) and a micromethod described by Witte and Matthaei (12). Each pineal was homogenized by sonication in 50 μl of chilled 0.05 *M* phosphate buffer, pH 6.8. Aliquots of 10 μl (=1/5 of a pineal gland) of this homogenate were incubated in the presence of 5.6 m*M* tryptamine–HCl (5 μl), 800 m*M* acetyl coenzyme A (2 μl), 0.765 nmole of acetyl-[1-^{14}C]coenzyme A (New England Nuclear: 52.3 mCi/mmole; 40 nCi/sample), and 1 μl phosphate buffer, pH 6.8, to yield a total incubation volume of 20 μl. Each sample was measured in duplicate. Incubation was continued for 20 min at 37°C and stopped by the addition of 100 μl of 0.2 *M* borate buffer, pH 10. The *N*-acetyl-[^{14}C]tryptamine formed was extracted with 1 ml chloroform and the aqueous phase removed by aspiration. The organic phase was then washed with 100 μl 0.2 *M* borate buffer, pH 10, and the aqueous phase again removed. A 0.5-ml aliquot of each chloroform extract was dispensed into a scintillation vial and allowed to dry. Radioactivity was quantified by liquid scintillation spectroscopy after adding 10 ml toluene/Liquifluor (1000:42). Results were expressed as nanomoles of *N*-acetyltryptamine formed per pineal per hour.

Hydroxyindole-O-methyltransferase assay. This assay is a modification of the microassay described by Witte and Matthaei (12) which is based on the assay of Axelrod and Weissbach (4). Each pineal gland was homogenized by sonication in 50 μl of chilled 0.05 *M* phosphate buffer, pH 7.9. Duplicates of 10-μl aliquots of the homogenate were incubated in the presence of 3 m*M* *N*-acetylserotonin (3 μl), 200 m*M* phosphate buffer, pH 7.9 (2 μl), 400 μ*M* *S*-adenosylmethionine (3 μl), *S*-adenosyl-L-[*Me*-^{14}C]-methionine (New England Nuclear: 56.2 mCi/mmole; 20 nCi/sample), and 1 μl distilled H_2O, to yield a total incubation volume of 20 μl for each duplicate. Incubation time was 30 min and the subsequent procedure was identical to the NAT assay. Results were expressed as nanomoles melatonin formed per pineal per hour.

Melatonin radioimmunoassay. Twenty-five microliters of the pineal homogenates (=1/2 pineal gland) were diluted to 500 μl with phosphate-buffered saline, pH 7.0, and duplicate 200-μl aliquots were assayed for melatonin content by the radioimmunoassay of Rollag and Niswender (13). The validity of using antiserum R1055 for the quantification of melatonin in the Syrian hamster pineal gland has been established previously.

Statistics. Results were expressed as means ± 1 standard error of the mean ($x \pm 1$ SEM) and were statistically analyzed using one-way analysis of variance followed by the Newman–Keuls multiple range test.

Results. NAT activity measured in 2-hr intervals over a period of 24 hr showed consistently low values during the light period and for the first 3 hr into the dark phase (mean = 0.13 nmole · pineal^{-1} · hr^{-1}). NAT activity was significantly elevated at 0300 and 0500 hr ($P < 0.001$); thereafter enzyme activity declined rapidly to low daytime values at 0700 hr (Fig. 1, top panel). No significant difference between NAT activity of male and female hamsters was observed and, therefore, the values from both sexes were combined.

HIOMT activity (Fig. 1, middle panel) was constant (Mean = 0.1 nmole · pineal^{-1} · hr^{-1}) throughout the 24-hr period and again no statistical differences between males and females were observed.

Melatonin content (Fig. 1, bottom panel) was low during the day, began to increase after 2300 hr, reached a peak value at 0300 hr, and fell to baseline levels by 0700 hr. In Fig. 2, the melatonin content of individual pineal glands was plotted as a function of their corresponding NAT activities. Neither during the day (Fig. 2a), nor at the time of NAT peak activity (Fig. 2b) could a significant correlation be detected between NAT activity and melatonin content of individual glands. The same lack of correlation is found between HIOMT activity and melatonin content.

Discussion. The assay procedure used in this study allows determination of enzyme activities in one-fifth of a Syrian hamster pineal gland. The values for NAT activity obtained by this method are consistent with earlier re-

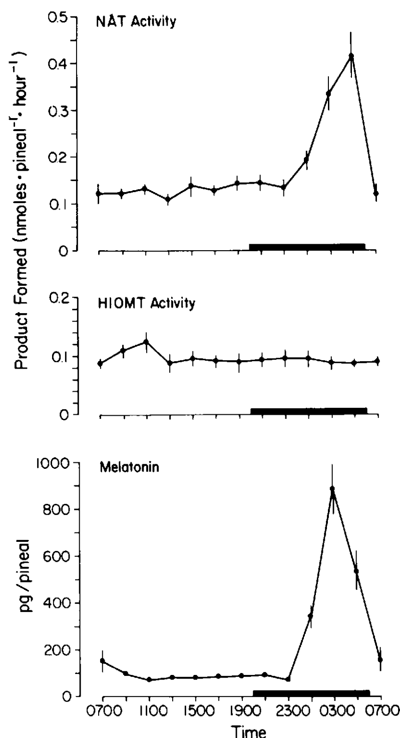

FIG. 1. Diurnal variation of NAT activity (nanomoles of *N*-acetyltryptamine formed per pineal per hour), HIOMT activity (nanomoles of melatonin formed per pineal per hour), and melatonin content (pg/gland) in the Syrian hamster pineal gland. Values of enzyme activities are means ± 1 SEM of at least 7 pineal glands measured in duplicate. Values for melatonin content are means ± 1 SEM of at least 14 pineal glands (duplicate determinations).

ports from this laboratory using a whole or one-half pineal gland (7, 8), but the data obtained can be considered more reliable since all samples were measured in duplicate.

This is the first report for HIOMT activity in the Syrian hamster pineal gland measured over a complete 24-hr period. No significant day/night rhythm was found. This is in agreement with previous reports (2, 15) where no difference in HIOMT activity was found between a pool of day and a pool of night pineal glands. The absolute values reported for HIOMT in these earlier studies, however, differ considerably from the values found in this report (25 pmole • pineal^{-1} • hr^{-1} vs 100 pmole • pineal^{-1} • hr^{-1}). The reason for this discrepancy is presumably related to the assay protocols, since Tamarkin and co-workers (2) incubated in phosphate buffer of pH 6.8 which is quite different from the optimal pH of 7.9 (4, 16) used in the present study. Axelrod *et al.* (5) originally reported daily changes in HIOMT activity of the rat pineal gland. However, these findings could not be confirmed by other laboratories (17, 18). More recent studies by Balemans and co-workers (19) again show a diurnal rhythm of HIOMT in the rat pineal gland if the enzyme is measured by a different technique.

The largest increase in melatonin content in the hamster pineal gland is about 230 pg/hr or roughly 1 pmole/hr. Since HIOMT activity is about 100 pmole • pineal^{-1} • hr^{-1}, 1/100 of this activity would produce the necessary increase in melatonin content, if no melatonin were secreted. Rollag *et al.* (20) calculated that a synthesis rate of 70.6 pg/min is required to account for the nocturnal peak in melatonin content, when corrected for melatonin release and/or degradation. This

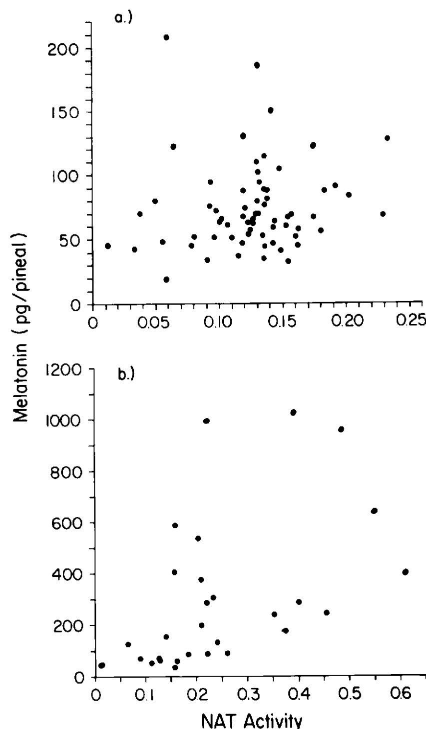

FIG. 2. Melatonin content of individual pineal glands plotted as a function of their corresponding NAT activities. (a) pineal glands collected from 0900 through 2300 hr; (b) pineal glands collected from 0100 through 0700 hr. Note different scale.

represents a synthesis rate of roughly 18 pmole/hr, which is still five times lower than the HIOMT activity measured.

However, calculations of this type need to be considered with caution since we do not know the actual enzyme activities *in vivo*. HIOMT activity, in the assay, is measured under artificial conditions *in vitro* with excess substrates provided and at the pH optimum for the reaction. In a previous experiment, hamster pineal *N*-acetylserotonin was below detectable levels (as measured by HPLC) in all samples, even at the time of peak NAT activity (Steinlechner *et al.*, unpublished observations). Hence, HIOMT may never be saturated with this substrate *in vivo*.

The same limitations apply for NAT activity. Therefore, the enzyme activities reported here are better termed "potential maximal activities under optimal *in vitro* conditions" and might be quite different from actual *in vivo* activities. It is notable that optimal pH values for NAT and HIOMT are considerably different (pH 6.8 and 7.9, respectively). NAT is especially sensitive to pH changes and may be irreversibly blocked by a pH higher than 6.9, while HIOMT only shows 30% of its maximal activity at this pH (16). For this reason, we were unable to measure NAT and HIOMT in the same pineal homogenate, although the volume would be sufficient for both assays. Both enzymes are soluble proteins located entirely in the cytoplasm of the pinealocytes (4, 21). In the intact cell, the enzymes either have to work at suboptimal pH or they have to be compartmentalized. This strong pH dependence may be one possible mechanism by which the enzyme activities *in vivo* are regulated; e.g., via oscillations of the intracellular pH.

Both NAT activity and melatonin concentration in the Syrian hamster pineal gland appear to follow the same temporal pattern with low values from 0700 through 2300 hr and elevated values for the remaining hours. Examination of Fig. 1 indicates that the peak values do not coincide. The highest value for melatonin content seems to be at 0300 hr whereas the highest measured peak of NAT activity is at 0500 hr, i.e., seemingly 2 hr after the melatonin peak. One could argue that the actual peaks for both rhythms occurred at 0400 hr and were missed in the present study. However, this does not account for the finding that the lower NAT activity at 0300 hr results in a higher melatonin content at 0300 hr compared to those values at 0500 hr. This lack of correlation becomes more apparent when the enzyme activities of single glands are compared with their corresponding melatonin contents (Fig. 2). We do know, however, that the exposure of Syrian hamsters to light at night has the same marked suppressive effect in both pineal NAT activity and melatonin levels (22). Considering the present finding, we draw attention to the lack of correlation between NAT activity and melatonin levels in the pineal of hamsters killed throughout a light:dark cycle. Furthermore, we caution against the assumption that NAT activity necessarily reflects the melatonin content of the hamster pineal gland.

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