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Detection of melatonin and serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase activities in rat ovary

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Abstract

Melatonin (*N*-acetyl-5-methoxytryptamine) and the activities of two melatonin-synthesizing enzymes, serotonin *N*-acetyltransferase (acetyl coenzyme A: arylalkylamine *N*-acetyltransferase EC 2.3.1.87; NAT) and hydroxyindole-*O*-methyltransferase (*S*-adenosyl-L-methionine: *N*-acetylserotonin-*O*-methyltransferase EC 2.1.1.4; HIOMT), were assayed in extracts of ovaries obtained from virgin Wistar-derived rats (7–9 week-old) during the light period of a 12 h light/12 h dark cycle. Melatonin was detected in the rat ovary using reverse-phase high-performance liquid chromatography (HPLC) coupled with fluorometric detection and radioimmunoassay (RIA). In addition, NAT and HIOMT activities were found in rat ovary. The apparent Michaelis constants (K_m) for the substrates of NAT and HIOMT in the rat ovary were similar to those reported for the pineal gland and retina. These data suggest that the rat ovary, like the pineal gland and the retina, may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT. © 1997 Elsevier Science Ireland Ltd.

Keywords: Melatonin; Serotonin *N*-acetyltransferase; Hydroxyindole-*O*-methyltransferase; Rat ovary

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine originally identified in the pineal gland, is synthesized from serotonin by the sequential action of serotonin *N*-acetyltransferase (acetyl coenzyme A: arylalkylamine *N*-acetyltransferase EC 2.3.1.87; NAT) and hydroxyindole-*O*-methyltransferase (*S*-adenosyl-L-methionine: *N*-acetylserotonin-*O*-methyltransferase EC 2.1.1.4; HIOMT) (Axelrod, 1974; Namboodiri et al., 1987; Sugden et al., 1987). The synthesis and secretion of melatonin display diurnal rhythms peaking during the dark period of 24 h light/dark cycles and a circadian rhythm under constant darkness (Axelrod, 1974; Tamarkin et al., 1985; Binkley, 1993; Brzezinski, 1997). It is widely accepted that melatonin is an endogenous mediator of photoperiodic information and a molecular component of the circadian timekeeping system (Axelrod, 1974; Tamarkin et al., 1985; Binkley, 1993; Reiter, 1993; Dollins et al., 1994; Brzezinski, 1997).

It has been demonstrated that melatonin is synthesized in extrapineal tissues such as the retina and intestine of mammals (Huether, 1993; Tosini and Menaker, 1996). In these tissues, melatonin appears to function as a local regulator of various aspects of rhythmic activity (Lergris et al., 1982; Besharse and Dunis, 1983; Harlow and Wekly, 1986) and as a neuromodulator (Dubocovich, 1983).

Melatonin is also present in fluid from human preovulatory ovarian follicles (Brzezinski et al., 1987; Ronnberg et al., 1990). However, whether melatonin is present in the ovary of other mammalian species is unknown. In addition, it has been presumed that mela-

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tonin detected in the human follicular fluid may be derived from the general circulation, because ovaries of cats and rats take up and retain circulating [^{3}H]melatonin (Wurtman et al., 1964). However, the possibility of de novo synthesis of melatonin in the ovary has not been negated. We now report that melatonin and the activities of two melatonin-synthesizing enzymes, NAT and HIOMT, are present in the rat ovary. Also, the kinetic analysis using rat ovary homogenates indicates that the apparent Michaelis constants (K_m) for substrates of NAT and HIOMT are similar to those that have been reported in the pineal gland and retina. These findings suggest that the ovary, like the pineal gland and retina, may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT.

2. Materials and methods

2.1. Chemicals

All the reagents used were of the highest purity available. Melatonin, its structurally related compounds, acetyl coenzyme A, and *S*-adenosyl-L-methionine (SAM) were purchased from Sigma (St. Louis, MO). *N*-acetyltryptamine was synthesized from tryptamine and acetic anhydride (Itoh et al., 1995). For radioimmunoassay (RIA), [*O*-methyl-^{3}H]melatonin was obtained from Amersham (Buckinghamshire, UK) and anti-melatonin serum was from Professor K. Wakabayashi (Gunma University, Japan). The dye reagent used for protein assay was obtained from Bio-Rad.

2.2. Animals

The 7–9 week-old virgin Wistar-derived rats weighing 182–230 g were used. Rats were maintained in a 12 h light/12 h dark lighting schedule, with access to food and water ad libitum, and were killed by decapitation during the light period. Ovaries were rapidly removed, frozen on solid CO_2 or in liquid N_2, and stored at -80°C until assayed. The stage of the ovarian cycle from which the tissues were taken was not examined.

2.3. Sample preparation for melatonin determination

Ovaries obtained from four to six rats were homogenized in 1.0–1.5 ml of ice-cold 0.20 M sodium borate buffer (pH 10.0). The homogenate was extracted with 8–12 ml of chloroform. The chloroform phase was washed with 1 ml of distilled water and evaporated. The residue was stored at -20°C until melatonin determination by high-performance liquid chromatography (HPLC). The recovery of the extraction procedure was $81.4 \pm 3.6\%$ (mean $\pm$ SEM, $n = 4$).

2.4. Melatonin determination by HPLC

The dried residue was redissolved in 100 or 150 μl of the HPLC mobile phase consisting of 50 mM ammonium acetate buffer (pH 4.3) and 20% methanol (v/v), and was filtered through a 0.45 μm filter. The filtrate (30–50 μl) was applied to a chromatographic system equipped with a Superiox ODS S-5 μm column (4.6 × 150 mm, I.D.; Shiseido, Tokyo) and a fluorometric detector (RF-550, Shimadzu, Kyoto). The detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. All separations were carried out isocratically at a flow rate of 1.0 ml/min of the above mentioned HPLC mobile phase and at 30°C. The fraction that comigrated with authentic melatonin was collected. To identify the melatonin peak, the methanol concentration of the mobile phase was varied in the range of 12–25%, and authentic melatonin was added to samples and analyzed. Peaks were identified by retention times and melatonin was quantified by peak height. The limit of sensitivity of the assay was as low as 20 pg for a 2:1 signal-to-noise ratio.

2.5. Melatonin radioimmunoassay

Aliquots of the HPLC fraction corresponding to the authentic melatonin peak were extracted with four volumes of chloroform. The chloroform phase was evaporated and the residue was dissolved in 600 μl of 10 mM phosphate buffered saline (pH 7.4) containing 1% bovine serum albumin (BSA). The melatonin levels of serially diluted samples were determined by RIA as described in (Itoh et al. (1995)).

2.6. Serotonin N-acetyltransferase activity assay

NAT activity was assayed by measuring the amount of *N*-acetyltryptamine formed from tryptamine and acetyl coenzyme A (Thomas et al., 1990; Itoh et al., 1995). Single ovaries were each homogenized in 200 μl of ice-cold 0.25 M potassium phosphate buffer (pH 6.5) containing 1.4 mM acetyl coenzyme A. The homogenate was centrifuged at $12\,000 \times g$ for 20 min at 4°C and 75 μl of the supernatant was mixed with 25 μl of 8 mM tryptamine HCl in 0.25 M potassium phosphate buffer (pH 6.5). The mixture was incubated for various times at 37°C. The final concentrations of acetyl coenzyme A and tryptamine were 1.0 and 2.0 mM, respectively. The reaction was stopped by addition of 20 μl of 6 M perchloric acid. After centrifugation at $15\,000 \times g$ for 20 min at 4°C, the supernatant was filtered through a 0.45 μm filter and the filtrate (1–5 μl) was applied to a HPLC system equipped with the same column and fluorometric detector as those used for melatonin determination. The detector was used with the excitation and emission wavelengths set

at 285 and 360 nm, respectively. The mobile phase consisted of 50 mM phosphoric acid, 32% methanol (v/v) and 0.65 mM sodium octylsulfate, adjusted to pH 3.5 with NaOH, and was pumped at a flow rate of 1.0 ml/min. Peaks were identified by the retention time and *N*-acetyltryptamine was quantified by peak height. As controls, reaction mixtures were incubated without tryptamine or without enzyme source and were analyzed using HPLC. For kinetic analysis, tryptamine and acetyl coenzyme A were used at various concentrations.

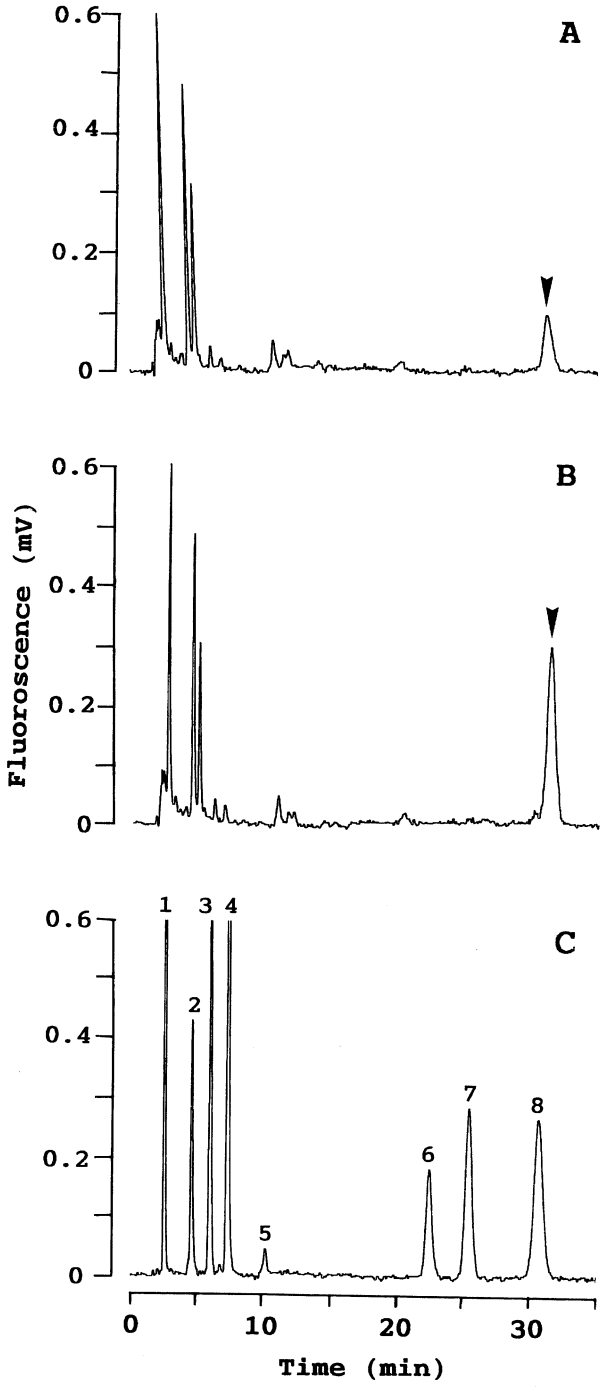

Fig. 1. (*Continued*)

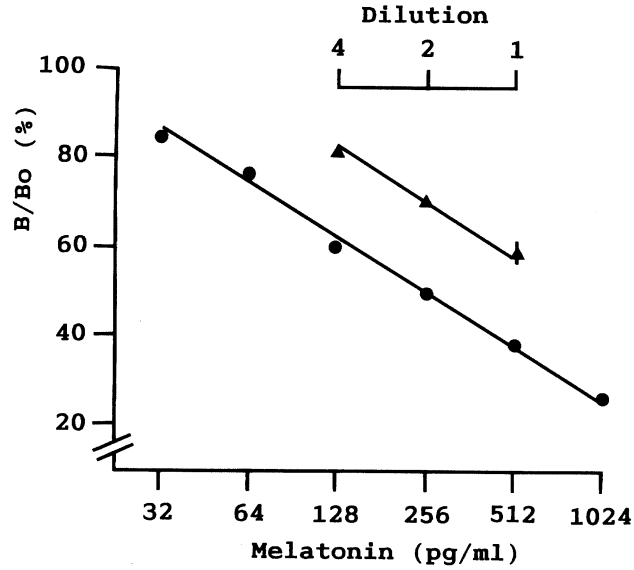

Fig. 2. Melatonin RIA inhibition curves for melatonin standard (●) and the fractions corresponding to the melatonin peak in the HPLC chromatograms of rat ovary extracts (▲). Each point and vertical line indicates the mean ± SEM of triplicate determinations

In addition, serotonin was used as the substrate, and *N*-acetylserotonin (NAS) formed enzymatically was detected by HPLC with fluorometric detection. The HPLC conditions were the same as for melatonin determination.

2.7. Hydroxyindole-O-methyltransferase activity assay

HIOMT activity was assayed by measuring the amount of melatonin formed from NAS and SAM (Itoh et al., 1997). Single ovaries were each homogenized in 200 μl of ice cold 50 mM sodium phosphate buffer (pH 7.9) followed by centrifugation (12 000 × *g* for 10 min at 4°C). Aliquots (55 μl) of the supernatant

Fig. 1. Representative chromatograms of (A) extracts of rat ovaries (B) the same samples spiked with 200 pg of authentic melatonin (*N*-acetyl-5-methoxytryptamine); and (C) a standard solution containing 250 pg of each of several indole compounds. The arrowheads in (A) and (B) indicate the elution position of melatonin. Peaks in (C): (1) 5-hydroxytryptamine (serotonin); (2) 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol; (3) NAS; (4) 5-methoxytryptamine; (5) 6-hydroxymelatonin; (6) 5-methoxyindole-3-acetic acid; (7) 5-methoxytryptophol; (8) melatonin. Ovaries were obtained from six female rats (7 week-old) during the light period of a 12 h light/12 h dark cycle and were homogenized in ice-cold 0.20 M sodium borate buffer (pH 10.0). After extraction with chloroform and evaporation, the residue was dissolved in 150 μl of the mobile phase of HPLC and filtered through a 0.45 μm filter. Filtrate (48 μl) or the filtrate spiked with authentic melatonin (50 μl) was subjected to reverse-phase HPLC with fluorometric detection. The above mentioned standard solution was also subjected to HPLC with fluorometric detection. The methanol concentration of the HPLC mobile phase was 20%. For other experimental conditions, see Materials and methods.

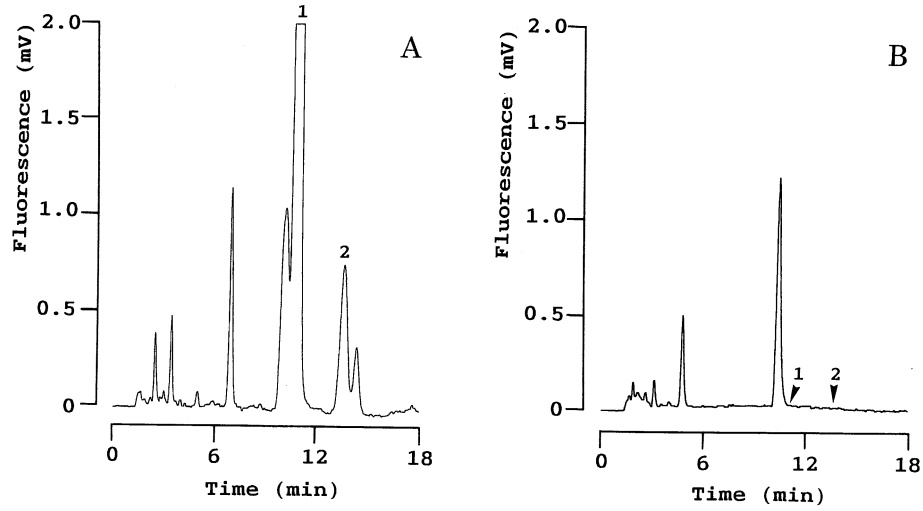

Fig. 3. Representative chromatograms of (A) rat ovary homogenates incubated with acetyl coenzyme A and tryptamine, and (B) rat ovary homogenates incubated with acetyl coenzyme A and without tryptamine. Peaks: (1) tryptamine and (2) *N*-acetyltryptamine. A single ovary (7 week-old) obtained during the light period of a 12 h light/12 h dark cycle was homogenized in 200 μl of ice-cold 0.25 M potassium phosphate buffer (pH 6.5) containing 1.4 mM acetyl coenzyme A. After centrifugation, 75 μl of the resulting supernatant was assayed for NAT activity (20 min incubation at 37°C). For other experimental conditions, see Materials and methods.

were mixed with 25 μl of 3.2 mM NAS and 0.32 mM SAM in 50 mM sodium phosphate buffer (pH 7.9) and incubated for various times at 37°C. The final concentrations of NAS and SAM were 1.0 and 0.1 mM, respectively. The enzyme reaction was stopped by the addition of 20 μl of 6 M perchloric acid. After centrifugation at 15 000 $\times$ *g* for 20 min at 4°C, the supernatant was filtered through a 0.45 μm filter. The filtrate (40–50 μ1) was subjected to the HPLC analysis. The HPLC conditions were the same as for melatonin determination. As controls, reaction mixtures were incubated either without NAS or without enzyme source, and were analyzed using HPLC. For kinetic analysis, NAS and SAM were used at various concentrations.

2.8. Protein assay

Protein content was determined by a dye-binding method with BSA as the standard (Bradford, 1976).

3. Results

In the rat ovary extracts, a peak with an identical retention time (30.8 min) to that of authentic melatonin was found by reverse-phase HPLC coupled with fluorometric detection (Fig. 1A). Peaks with retention times identical to those of other indole compounds including serotonin and NAS, which are melatonin precursors, were also detected on the HPLC chromatogram. The addition of known amounts (200 pg) of authentic melatonin to the ovary extracts increased the melatonin-like peak only (Fig. 1B).

Melatonin-like immunoreactivity was detected by RIA in the HPLC fraction that comigrated with authentic melatonin (Fig. 2). The displacement curve of the serially diluted fraction was parallel to that of the authentic melatonin standard. The melatonin content

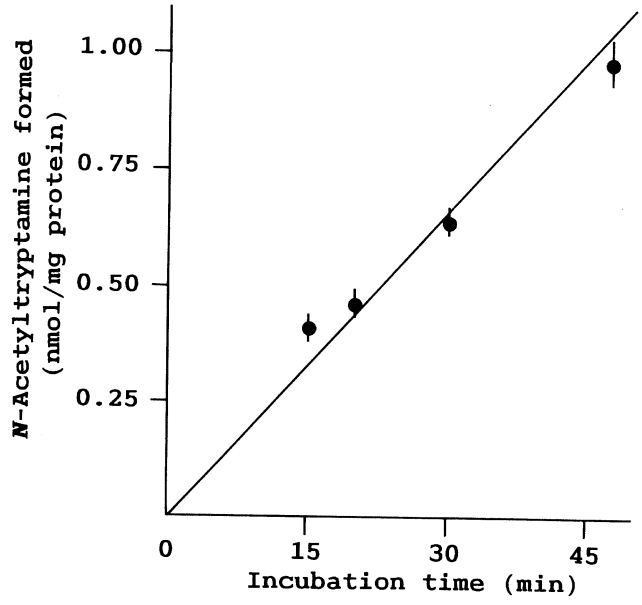

Fig. 4. Time course of *N*-acetylation of tryptamine using rat ovary homogenate as the enzyme source. Ovaries were obtained from five rats (7–9 week-old) during the light period of a 12 h light/12 h dark cycle and homogenized in 1.2 ml of ice-cold 0.25 M potassium phosphate buffer (pH 6.5) containing 1.4 mM acetyl coenzyme A. The enzyme reaction was carried out for various times at 37°C. For other experimental conditions, see Materials and methods. Each point and vertical line indicates the mean $\pm$ SEM of triplicate determinations.

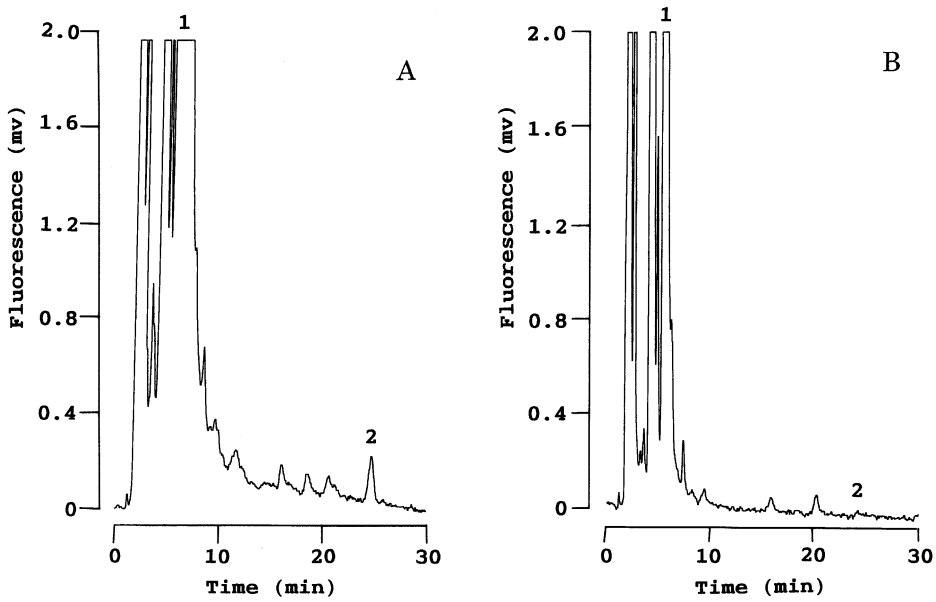

Fig. 5. Representative chromatograms of (A) rat ovary homogenates incubated with SAM and NAS and (B) rat ovary homogenates incubated with SAM and without NAS. Peaks in (A) and (B) were (1) NAS and (2) melatonin. A single ovary (7 week-old) obtained during the light period of a 12 h light/12 h dark cycle was homogenized in 200 μl of ice-cold 50 mM sodium phosphate buffer (pH 7.9). After centrifugation, 55 μl of the resulting supernatant was assayed for HIOMT activity (1 h incubation at 37°C). The methanol concentration of the HPLC mobile phase was 22%. For other experimental conditions, see Materials and methods.

(mean $\pm$ SEM, $n = 5$) of rat ovaries obtained during the light period of a 12 h light/12 h dark cycle was 48.5 $\pm$ 5.2 pg/ovary by HPLC determination and 44.3 $\pm$ 4.7 pg/ovary by RIA.

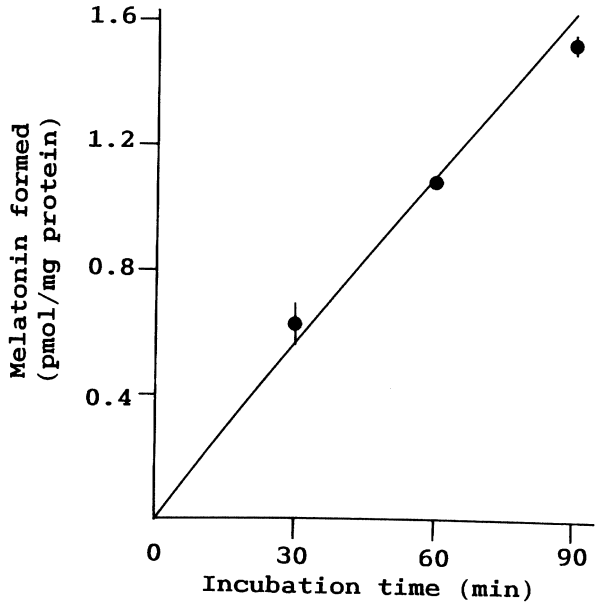

Fig. 6. Time course of *O*-methylation of NAS using rat ovary homogenate as the enzyme source. Ovaries were obtained from eight rats (7–9-week-old) during the light period of a 12 h light/12 h dark cycle and homogenized in 1.5 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.9). The enzyme reaction was carried out for various times at 37°C. For other experimental conditions, see Materials and methods. Each point and vertical line indicates the mean $\pm$ SEM of triplicate determinations.

When rat ovary homogenate was incubated with tryptamine and acetyl coenzyme A, an *N*-acetyltryptamine peak was detected using HPLC analysis (Fig. 3A). No *N*-acetyltryptamine peak was detected in control incubations without tryptamine (Fig. 3B) or without enzyme source (data not shown). The rate of *N*-acetyltryptamine formation proceeded linearly for 45 min at 37°C (Fig. 4). The NAT activity (mean $\pm$ SEM, $n = 7$) of the rat ovaries was 5.26 $\pm$ 0.15 or 1.38 $\pm$ 0.04 nmol/h per mg protein.

Fig. 5 shows the HPLC chromatograms obtained from reaction mixtures of rat ovary homogenate with NAS and SAM (experimental incubation) or without NAS (control incubation). The melatonin peak was higher in the experimental incubation than in the control incubation. In mixtures of substrates without the enzyme source, no melatonin peak was detected using HPLC analysis (data not shown). The rate of melatonin formation proceeded linearly for 90 min at 37°C (Fig. 6). The HIOMT activity (mean $\pm$ SEM, $n = 5$) of the rat ovaries was 4.14 $\pm$ 0.23 or 1.11 $\pm$ 0.06 pmol/h per mg protein.

A kinetic analysis of rat ovary NAT for two substrates, tryptamine and acetyl coenzyme A, was carried out. The apparent K_m values (mean $\pm$ SEM, $n = 3$) as determined by Lineweaver-Burk plots were 3.3 $\pm$ 0.6 mM for tryptamine and 0.18 $\pm$ 0.02 mM for acetyl coenzyme A (Fig. 7). When serotonin was used as the substrate in NAT activity assay, the apparent K_m value (mean $\pm$ SEM, $n = 3$) for serotonin was 2.3 $\pm$ 0.8 mM.

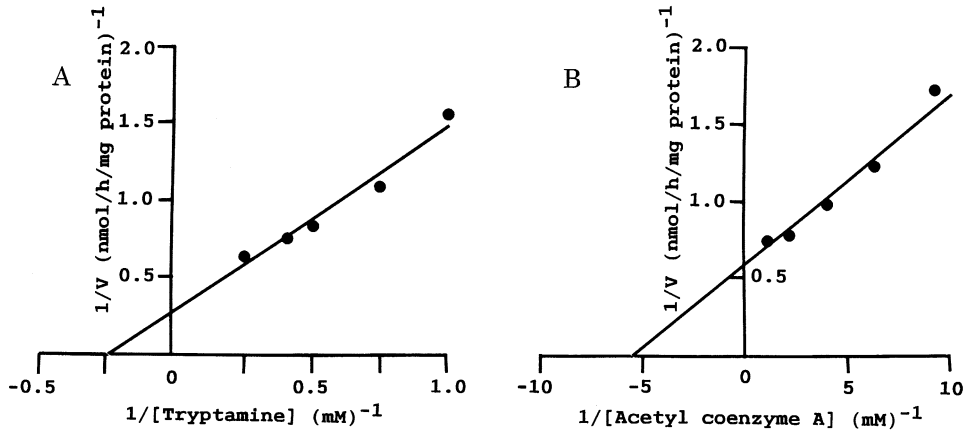

Fig. 7. Lineweaver-Burk plots of rat ovary NAT for (A) tryptamine and (B) acetyl coenzyme A. Ovaries were obtained from ten rats (7–9 week-old) during the light period of a 12 h light/12 h dark cycle and homogenized in 2.4 ml of ice-cold 0.25 M potassium phosphate buffer (pH 6.5). After centrifugation, the resulting supernatant (protein concentration 31.7 μg/μl) was assayed for NAT activity (20 min incubation at 37°C). To determine the Michaelis constant (K_m) for tryptamine, the acetyl coenzyme A concentration was fixed at 1.2 mM. For the acetyl coenzyme A K_m determination, the tryptamine concentration was 1.0 mM. All points represent the mean of duplicate determinations, which differed by $< 10\%$. The K_m values were determined by linear regression analysis. The data shown are representative of three experiments.

A kinetic analysis of the rat ovary HIOMT for NAS and SAM was carried out . The apparent K_m values (mean $\pm$ SEM, $n = 3$) as determined by Lineweaver-Burk plots were 0.04 ± 0.01 mM for NAS and 0.04 ± 0.02 mM for SAM (Fig. 8).

4. Discussion

The results of this study clearly show that melatonin is present in the rat ovary, because a peak with an identical retention time to that of authentic melatonin was found in rat ovary extracts by reverse-phase HPLC coupled with fluorometric detection (Fig. 1) and melatonin-like immunoreactivity was detected in the HPLC fraction corresponding to melatonin's retention time (Fig. 2). It is possible that melatonin detected in the ovary is derived from circulation, because the ovary takes up and retains circulating [^{3}H]melatonin (Wurtman et al., 1964). However, the present data suggest another possibility that the rat ovary may synthesize melatonin from serotonin by the sequential action of NAT and HIOMT for the following reasons: (i) NAT and HIOMT activities were detected in rat ovary homogenates (Figs. 3 and 5); (ii) the apparent K_m values for substrates of NAT and HIOMT in the rat ovary were similar to those reported for the pineal gland and the retina (Figs. 7 and 8) (Cardinali and Wurtman,

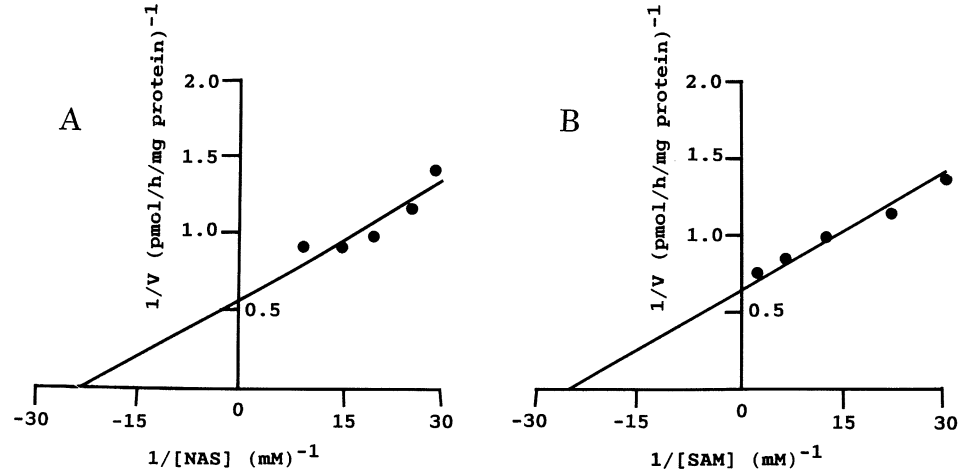

Fig. 8. Lineweaver-Burk plots of rat ovary HIOMT for (A) NAS and (B) SAM. Ovaries were obtained from 15 rats (7–9 week-old) during the light period of a 12 h light/12 h dark cycle and homogenized in 3.0 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.9). After centrifugation, the resulting supernatant (protein concentration 37.4 μg/μl) was assayed for HIOMT activity (1 h incubation at 37°C). To determine the Michaelis constant (K_m) for NAS, the SAM concentration was fixed at 0.1 mM. For the SAM K_m determination, the NAS concentration was 1.0 mM. All points represent the mean of duplicate determinations, which differed by less than 10%. The K_m values were determined by linear regression analysis. The data shown are representative of three experiments.

1972; Deguchi, 1975; Sugden and Klein, 1983; Voison et al., 1984), strongly suggesting that rat ovary NAT and HIOMT function physiologically as melatonin-synthesizing enzymes; (iii) the HPLC chromatogram of rat ovary extracts showed peaks with identical retention times to those of serotonin and NAS, which are melatonin precursors (Fig. 1).

To obtain further evidence that a melatonin-synthesizing system is present in the rat ovary, it is necessary to examine whether the melatonin-synthesizing enzymes and their substrates are found in the same cells or tissue compartments and what proportion of the ovarian melatonin is derived from local production.

It has been reported that a putative melatonin receptor is present in the rat ovary (Cohen et al., 1978), and that melatonin induces the decrease in the ovarian weight (Wurtman et al., 1963) and the increase in progesterone production by ovarian granulosa cells of rats in vitro (Fiske et al., 1984). Therefore, it is possible that melatonin synthesized in the ovary may regulate reproduction at the follicular level. The significance of melatonin in the ovary should be further investigated.

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